Novel Insights into the Pathophysiology of Kidney Disease in Methylmalonic Aciduria

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DECLARATION

I hereby declare that the presented work and results are the product of my own work. Contributions of others or sources used for explanations are acknowledged and cited as such.

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Peer-reviewed publications presented in this work:

Haarmann A, Mayr M, Kölker S, Baumgartner ER, Schnierda J, Hopfer H, Devuyst O, Baumgartner MR. Renal involvement in a patient with cobalamin A type (cblA) methylmalonic aciduria: a 42-year follow-up. Mol Genet Metab. 2013 Dec;110(4):472-6. doi: 10.1016/j.ymgme.2013.08.021. Epub 2013 Sep 17.

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Peer-reviewed publications associated with the thesis:

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This thesis is dedicated to everyone who supported me on my way.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AdoCbl	5’-deoxyadenosylcobalamin, Adenosylcobalamin
ATP	Adenosine Triphosphate
BfnAI	Bafilomycin
BCAA	Branched chain amino acids
BCKD	Branched chain 2-ketoacid dehydrogenase complex
cbl	Cobalamin
CKD	Chronic kidney disease
CoA	Acyl coenzyme A
C3	Propionyl carnitine
CoQ_{10}	Coenzyme Q_{10}
CtsD	Cathepsin D
DT	Distal tubule
DNA	Desoxyribonucleic aid
GC-MS	Gas chromatography mass spectometry
GFR	Glomerular filtration rate
IEM	Inborn errors of metabolism
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IVA	Isovaleric aciduria
KIM-1	Kidney injury molecule-1
LCN2	Lipocalin 2
Mitoquinone	MitoQ
MQC	Mitochondrial quality control
MMA	Methylmalonic aciduria
MMP	Mitochondrial membrane potential
mPTC	Mouse proximal tubule cells
mROS	Mitochondrial reactive oxygen species
MS	Methionine synthase

MS/MS	Tandem mass spectometry
mtDNA	Mitochondrial DNA
MSUD	Maple sirup urine disease
MT	MitoTEMPO
MUT	Methylmalonyl-CoA mutase
Mut(-)	residual enzyme activity of MUT
Mut(0)	no residual enzyme activity of MUT
OA	Organic aciduria
OMM	Outer mitochondrial membrane
PA	Propionic aciduria
PT	Proximal tubule
PGC-1-α	Peroxisome proliferator-activated receptor-gamma coactivator-alpha
ROS	Reactive oxygen species
TAL	Thick ascending limb
TCA	Tricarboxylic acid cycle (=Krebs-cycle)
TGF-β	Transforming growth factor-beta

ABSTRACT IN ENGLISH

Disturbances in mitochondrial energy metabolism lead to severe effects on human health. To fulfill their important functions, mitochondria are organized in an interconnected network, providing energy and biosynthetic intermediates necessary for cellular homoeostasis and tissue function.

Methylmalonic aciduria (MMA) is a rare inborn error of metabolism due to the mutation of the mitochondrial enzyme methylmalonyl-CoA mutase (MUT) catalyzing the conversion of methylmalonyl-CoA to succinyl-CoA requiring vitamin B_{12} as an essential co-factor. The reaction fuels metabolites from the break-down of branched chain amino acids, odd chain fatty acids, cholesterol and propionic acid into the Krebs cycle (TCA). Mutations in the recessively inherited *MUT*-gene lead to a partial mut(-) or complete mut(0) loss of MUT function. MMA clinically results in severe metabolic acidosis due to accumulation of organic acids (predominantly methylmalonic acid), hyper-ammonaemia and an acute, life-threatening metabolic crisis usually manifesting in the neonatal period.

MUT is expressed in all tissue organs; high expression levels are found in the kidney, where mitochondria provide energy for transport processes mediated by renal tubular cells. Renal tubular dysfunction and chronic kidney disease (CKD) due to tubulo-interstitial lesions are the main long-term consequence in MMA patients. The pathomechanisms linking mitochondrial enzyme deficiency to renal epithelial dysfunction in basically all MMA patients remains largely unknown. Onset and progression of CKD seems to be linked to the severity of the mutation and is most pronounced in mut(0) patients, showing the highest levels of methylmalonic acid. Main hypothesis for CKD in MMA are abnormal mitochondrial function due to energetic depletion of the TCA, oxidative stress and potentially toxic effects of accumulating methylmalonic acid.

On top of suffering from a life-threatening disease, CKD imposes a high morbidity and mortality risk on MMA patients. The identification of kidney-protective measures and the in-depth understanding of the underlying pathomechanisms inducing CKD is crucial to slow down or even prevent severe kidney damage in a next step. Therefore, the aim of the thesis was to 1) investigate the stabilizing effect of co-factor application for renal protection in a mildly affected MMA patient and 2) to provide a (potentially targetable) link between MUT deficiency, mitochondrial dysfunction and renal epithelial cell damage in different *in vitro* and *in vivo* models.

The obtained data provide evidence, that even mild forms of MMA inevitably lead to CKD and underline the importance of early and adequate co-factor supplementation in vitamin B_{12}-responsive MMA patients. Maintenance of mitochondrial integrity is a key requirement to preserve cellular homeostasis and function particularly in highly specialized renal epithelial cells. Using different MMA-models we provide evidence, that MUT deficiency leads to severe disturbance of the mitochondrial network in terms of morphology, function and homeostasis. Dysregulation of the

organelle-specific mitochondrial quality control (MQC) system initiated by PINK1 and parkin induces impaired autophagy-mediated clearance of dysfunctional mitochondria. As a consequence, the cells accumulate autophagic proteins and mitochondrial reactive oxygen species potentiating cellular and tissue damage. Mitochondria-targeted treatment with the anti-oxidant Mito-TEMPO restores mitochondrial integrity and homeostasis, improves organelle-specific targeting by PINK1 and induces autophagic degradation of dysfunctional mitochondria. These changes lead to a reduction of the cellular damage process.

The results provide a link between mitochondrial dysfunction and kidney damage in MMA and highlight the importance of the MQS for mitochondrial integrity and homeostasis. Mitochondrial-targeted treatment offers new therapeutic options to address renal epithelial damage down-stream to primary mitochondrial diseases.

ABSTRACT IN GERMAN

Störungen des mitochondrialen Energiestoffwechsels führen beim Menschen zu schwerwiegenden gesundheitlichen Komplikationen. Um wichtige Funktionen wie Energiebereitstellung und Generierung von intrazellulären Botenstoffen ausführen zu können, sind Mitochondrien in eng verknüpften Netzwerken organisiert, welche Homöostase und Gewebsfunktion aufrechterhalten.

Methylmalonazidurie (MMA) ist eine seltene, angeborene Stoffwechselkrankheit, welche durch Mutationen des mitochondrialen Enzyms Methylmalonyl-CoA Mutase (MUT) verursacht wird. MUT ist unter Verwendung des essentiellen Co-Faktors Vitamin B_{12} für die Umwandlung von Methylmalonyl-CoA in Succinyl-CoA verantwortlich. Durch die Reaktion werden Metabolite, welche aus dem Abbau verzweigtkettiger Aminosäuren, ungeradzahliger Fettsäuren, Cholesterol und Propionsäure entstanden sind, in den Krebs-Zyklus eingespeist. Mutationen im autosomal-rezessiv vererbten *MUT*-Gen führen entweder zu einem partiellen mut(-) oder kompletten Verlust mut(0) der MUT-Enzymaktivität. Klinisch manifestiert sich MMA klassischerweise in der neonatalen Periode mit einer schwerwiegenden metabolischen Azidose (bedingt durch die Akkumulation organischer Säuren, vorwiegend Methylmalonsäure), sowie Hyperammonämie und einer akuten, lebensbedrohlichen metabolischen Krise.

MUT ist in allen Geweben des menschlichen Körpers exprimiert. Hohe Konzentrationen finden sich insbesondere in der Niere, wo Mitochondrien Energie für Transportprozesse liefern, welche von den renalen Tubulusepithelzellen ausgeführt werden. Renale tubuläre Dysfunkion und chronische Niereninsuffizienz (CNI) bedingt durch tubulo-interstitielle Läsionen sind die hauptsächlich beobachteten Langzeitkomplikationen bei MMA-Patienten. Der Pathomechanismus, welcher für die Verknüpfung des mitochondrialen Enzymmangels mit der renalen, epithelialen Dysfunktion bei faktisch allen MMA-Patienten verantwortlich ist, ist weitgehend unbekannt. Beginn und Progression der CNI scheinen mit der Schwere der Mutation verknüpft zu sein und werden daher am häufigsten bei Mut(0) Patienten gefunden, welche auch die höchste Konzentration an Methylmalonsäure aufweisen. Als Hauptursache für CNI bei MMA wird eine mitochondriale Dysfunktion angesehen, welche durch energetische Verarmung des Krebs-Zyklus, akkumulierenden oxidativen Stress sowie potentiell toxische Effekte der anflutenden Methylmalonsäure verursacht und unterhalten wird.

Das ohnehin erhöhte Morbiditäts- und Mortalitätsrisiko erfährt durch die CNI bei MMA-Patienten eine zusätzliche Steigerung. Die Identifizierung von Nieren-protektiven Maßnahmen und das eingehende Verständnis der der CNI zugrundeliegenden Pathomechanismen sind daher essentiell, um in einem nächsten Schritt eine Nierenschädigung zu verlangsamen oder sogar zu verhindern. Das Ziel der vorliegenden Arbeit war daher 1) die stabilisierende Wirkung der Co-Faktor Applikation auf die Nierenfunktion bei einem mild betroffenen, Vitamin B_{12}-responsiven MMA-Patienten zu evaluieren sowie 2) zu untersuchen, ob eine (potentiell behandelbare) Verbindung zwischen der Defizienz von

MUT, mitochondrialer Dysfunktion und Nierenepithelzellschädigung in verschiedenen *in vitro*- und *in vivo*- Modellen herzustellen ist.

Die erhobenen Daten belegen, dass auch milde Formen von MMA unweigerlich zu CNI führen und unterstreichen die Bedeutung einer frühen und adäquaten Co-Faktor Supplementierung in Vitamin B_{12}-responsiven MMA-Patienten. Die Aufrechterhaltung mitochondrialer Integrität ist -insbesondere in hoch spezialisierten Nierenepithelzellen- eine wesentliche Voraussetzung für die Erhaltung von zellulärer Homöostase und Funktion. Unsere Daten belegen, dass MUT-Defizienz zu einer erheblichen Störung des mitochondrialen Netzwerkes in Bezug auf Morphologie, Funktion und Homöostase führt. Eine Dysregulation der Organell-spezifischen mitochondrialen Qualitätskontrolle (MQK), welche durch PINK1 und Parkin mediiert wird, führt zu einem reduzierten Abbau dysfunktionaler Mitochondrien über Autophagie. Infolgedessen kommt es zu einer Akkumulation von Autophagie-spezifischen Proteinen und mitochondrialem oxidativen Stress, welche zu Zell- und Gewebeschäden führen. Eine zielgerichtete Behandlung der Mitochondrien mit dem Anti-Oxidans MitoTEMPO stellt die mitochondriale Integrität und Homöostase wieder her, verbessert das Organell-spezifische Targeting durch PINK1 und induziert den Abbau dysfunktioneller Mitochondrien durch Autophagie. Die beschriebenen Effekte führen zu einer Reduktion der zellulären Schädigung.

Die Ergebnisse weisen auf einen kausalen Zusammenhang zwischen mitochondrialer Dysfunktion und Nierenschäden bei MMA-Patienten hin und heben die Bedeutung der MQK für mitochondriale Integrität und Homöostase hervor. Eine zielgerichtete Behandlung der Mitochondrien mit einem Anti-Oxidans bietet neue therapeutische Optionen, um renale epitheliale Schäden zu vermindern, welche durch primäre mitochondriale Erkrankungen verursacht werden.

I. INTRODUCTION

I.1 Inborn errors of metabolism

I.1.1 Rare diseases

A disease is considered as "rare" in Europe, when it affects less than 1:2000 individuals. More than 7000 rare diseases, most of them of genetic origin, are currently identified. If the total patient number for each rare disease is marginal, taken together more than 30 million subjects are presenting with a rare disease. More than half of rare diseases affect children of which 35% will not celebrate their 5th birthday (EU commission for rare diseases, 2008).

Diagnoses of rare diseases are often delayed and complicated by variable prevalence between different populations and clinical heterogeneity: The age of onset, severity and progression of symptoms may be very different in patients and even siblings suffering from the same disease caused by identic mutations (Devuyst, 2014). Insufficient knowledge on ontology may also complicate prognosis and diagnosis. Different mutations may influence the same biological pathways and give rise to similar clinical, biochemical, and histopathological features, like e.g. in Fanconi's syndrome. Another complicating aspect is the absence of valid biomarkers for diagnosis and follow-up (Devuyst, 2014). Even if treatment is available a monitoring of effects (and conclusions from the observations) is made impossible. This may not only delay treatment of potentially treatable diseases but also hampers the patients' quality of live due to chronic, progressive and often live-threatening aspects of the disease (EU commission for rare diseases, 2008).

To improve the patients' situation, a combination of multi-level approaches has to be applied: Networks and registries need to collect patient-related information and disease courses. Omics technologies are needed to screen patient's samples for novel biomarkers which can be used for diagnosis and follow-up. Next-generation sequencing will help to simultaneously investigate all relevant genes for a phenotype. To link disease genes and clinical phenotypes, efficient model organisms (e.g. mouse, zebrafish) and appropriate cellular systems to study disease-related mechanisms are needed. The combination of different approaches will lead to a broader understanding of the underlying defects, a better understanding of the disease causing pathomechanisms and hopefully to identification of potential new treatment options.

I.1.2 Classification of inborn errors of metabolism

Inborn errors of metabolism (IEM) cause hereditary metabolic diseases and belong to the group of rare diseases. The overall incidence of patients affected by a metabolic disease is around 40/100.000 (Applegarth, 2000). Sub-groups may go down to 1:100.000 and lower.

IEM classically are monogenic diseases leading to insufficiency or complete loss of enzymatic activity in the affected pathway. As consequence, metabolites up-stream of the deficient enzyme accumulate and may cause disease manifestations whereas down-stream depletion of important substrates and intermediary products may also lead to deleterious effects. Most IEMs are inherited in an autosomal-recessive mode which explains why they are particularly common in societies with a high percentage of consanguinity (Al Jasmi, 2015). Few of them are X-linked affecting exclusively males or few females due to unfavorable X-inactivation. *De novo* mutations in the affected patient can occur. IEM are structurally caused by small scale (e.g. missense and nonsense mutations) and large-scale mutations (particularly large deletions). Ethnic predomination may be observed in sub-groups like e.g. Ashkenazi Jews and Finns (Jimenez-Sanchez, 2014).

The pattern of tissue-specific gene expression is different for each IEM. Some affected genes might be widely expressed ("-house-keeping genes-") while others fulfill specific tasks in highly specified tissues (e.g. rhodopsin in photoreceptors in the eye). 70% of the genes altered in IEM lead to a multi-systemic disease reflecting how tightly integrated and interconnected metabolic pathways are (Jimenez-Sanchez, 2014).

I.1.2.1 Presentation of inborn errors of metabolism

Inborn errors of metabolism can present at any age and often show multi-system involvement. That is why they are often divided into acute onset (within the neonatal period), chronic intermittent form ("on-/off"-phenomenon at any age) and chronic progressive form (onset and progression at any age). However, there are characteristic diagnostic criteria and typical time points of manifestation for IEMs and that is why they are often grouped in into 5 categories according to their manifestation (Fig. 1) (Hoffmann, 2010):

a) Intoxication type (aminoacidopathies, organic acidurias, urea cycle defects, galactosaemia, hereditary fructose intolerance)

Patients are usually born after an uneventful pregnancy usually without any specific stigmas. They are typically asymptomatic within the first hours or days of life. After exposure to the substrate that cannot be processed due to the IEM (certain amino acids, proteins and sugars), affected children start to accumulate toxic molecules and metabolites which they cannot break down therefore leading to "intoxication". Down-stream of the enzymatic block the system gets depleted and substrates for important intermediary pathways involved in energy production lack substrate. This leads to catabolism, further deterioration of the system and progression to a live-threatening metabolic crisis. Any condition resulting in catabolism (e.g. prolonged fasting, infections, fever, vaccination and

hormonal changes (growth, puberty, steroid therapy)) as well as overload with the un-metabolizable substrates (e.g. proteins, certain sugars) can induce another crisis.

b) Reduced fasting tolerance (fatty acid oxidation, disorders of ketogenesis, glycogen storage disease type I, disorders of glyconeogenesis)
These disorders usually present in fasting states or conditions with high energy demands. They may present initially as intoxication type diseases but more likely emerge in the second half of infancy (6-12 months) during infections with severe hypoglycaemia and acidosis.

c) Disturbed energy metabolism (mitochondrial disorders, long-chain fatty acid oxidation defects)
Disorders of disturbed energy metabolism usually present at birth but may present at any life time, in dependence of the organ involvement, the defect and the mode of X-inactivation. Mitochondrial disorders characteristically show multi-organ involvement which are classically triggered by changes in the carbohydrate intake or infections (e.g. certain viruses). Marked and rapid deterioration with irreversible loss of capabilities are frequent in such episodes. Highly energy dependent organ systems (e.g. brain, heart, kidneys, liver) are affected in particular.

d) Disturbed metabolism of complex molecules (peroxisomal disorders, lysosomal disorders, disorders of glycosylation)
Disorders of complex molecules usually have a creeping course often starting at birth with increasing signs and symptoms (e.g. organ enlargement and loss of organ function) as time goes by. In contrary to the before mentioned, there are no metabolic crisis or acute derailments.

e) Disturbed neurotransmission (non-ketotic hyperglycinaemia, sulfite oxidase deficiency, vitamin B_6 and pyridoxal-phosphate dependent seizures, GABA transaminase deficiency)
Patients suffering from disturbed neurotransmission usually present immediately after birth or even ante-partum showing intra-uterine seizures. The symptoms are the expression of disturbed neurotransmitter production, up-take, recycling or processing and receptor/transporter expression. There is no external trigger promoting or provoking the symptoms.

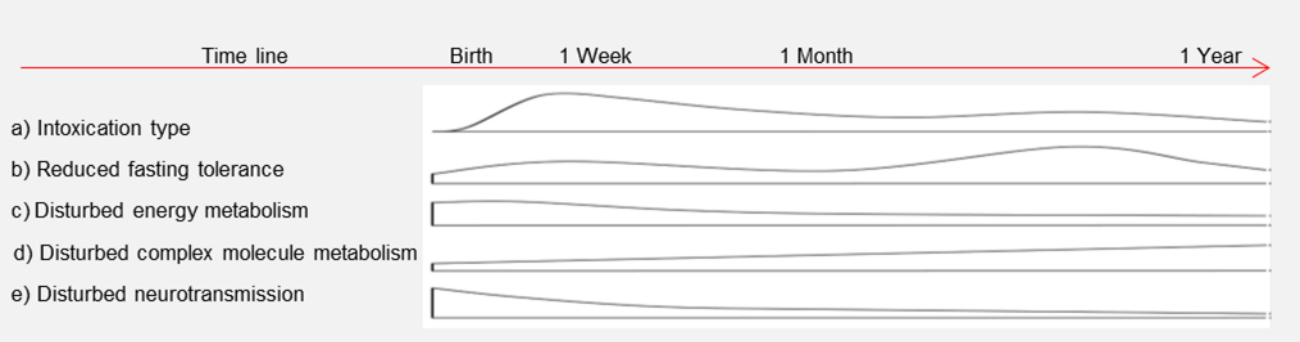

Figure 1: Typical age of disease manifestation and course in IEM. (Adapted from Hoffmann, 2010).

Among the above, the intoxication type diseases manifest with acute life-threatening metabolic crisis due to severe disturbance of metabolic energy metabolism and are causally treatable by fast intervention, if diagnosed in time. Defects in the break-down of branched chain amino acids causing organic acidurias are of particular interest, since they affect the mitochondrion as compartment and have a direct impact on cellular energy production.

I.2 Disorders of branched chain amino acid metabolism

I.2.1 Organic acidurias

Disorders of branched chain amino acid metabolism are caused by mutations of the enzymes involved in the breakdown of the branched chain amino acids (BCAA) leucine, isoleucine and valine leading to the disturbance of intermediary metabolism. The accumulation of characteristic organic acids leads to the diagnosis and that is why they are also called organic acidurias (OA).

The breakdown of the BCAAs begins with the degradation of essential amino acids to non-essential amino acids by transamination (Fig. 2). The process results in the generation of the corresponding 2-oxo-branched chain organic acids which are processed to branched chain acyl coenzyme A (CoA) products by oxidative decarboxylation. The second step is similar in the process of breakdown of all BCAAs and catalyzed by the branched-chain 2-ketoacid dehydrogenase complex (BCKDH). Afterwards, the pathways diverge: Leucine is cleaved down to isovaleryl-CoA, finally entering the TCA as acetyl-CoA. Isoleucine is processed to 2-methylbutyryl-CoA and can be converted to both acetyl- and succinyl-CoA. Valine forms isobutyryl-CoA finally leading to propionyl-CoA and succinyl-CoA providing substrate for the tricarboxylic acid cycle (TCA). Propionyl-CoA is also produced from the breakdown of the amino acids methionine and threonine as well as from fatty acids with an odd number of carbons and resident bacteria from the gut. (Vockley, 2014).

Defects in this pathway cause the 13 different organic acidurias known today. The most frequent OAs (maple sirup urine disease (MSUD), isovaleric aciduria (IVA), propionic aciduria (PA) and methylmalonic aciduria (MMA) are caused by disturbance of the intra-mitochondrial breakdown of BCAA and lead to accumulation of characteristic CoA derivatives and organic acids.

Other rare disorders of BCAA metabolism are 3-methylcrotonyl-glycinuria, 3-methylglutaconic aciduria, short-/branched chain acyl CoA dehydrogenase deficiency, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency, isobutyryl-CoA dehydrogenase deficiency, 3-hydroxy-isobutyric aciduria and malonic aciduria (Vockley, 2014), which will not be discussed in this thesis.

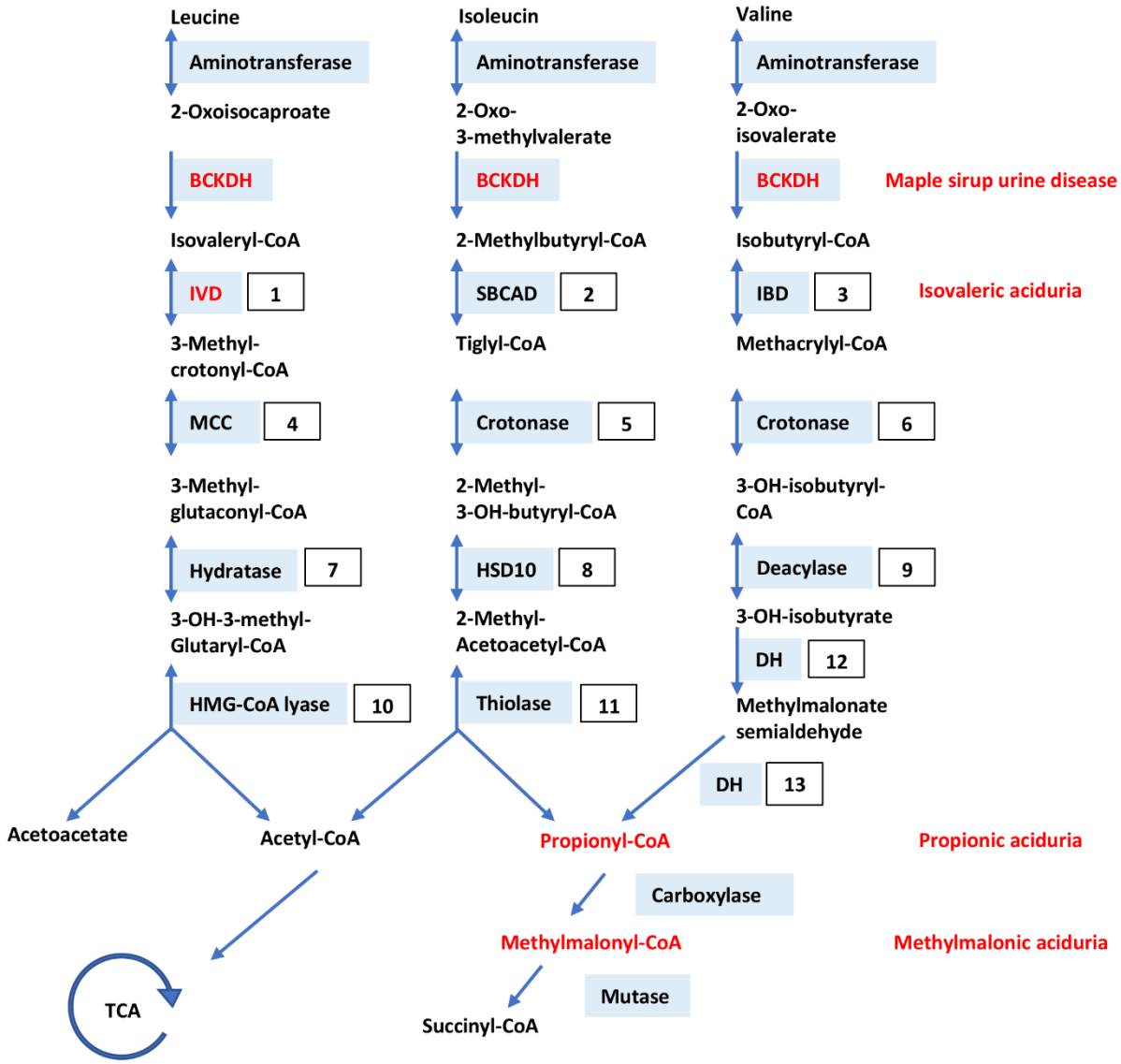

Figure 2: Summary of branched chain amino acid metabolism. Enzymes involved in the catabolism of leucine, isoleucine, and valine are shown in blue boxes. The 13 different organic acidurias known today are numbered and start at the level of the branched chain acyl-CoA dehydrogenases (1, isovaleryl-CoA dehydrogenase; 2, short/branched chain acyl-CoA dehydrogenase; and 3, isobutyryl-CoA dehydrogenase). The remaining enzymes in the pathway are 4, 3-methylcrotanyl-CoA carboxylase; 5, tiglyl-CoA hydratase; 6, methacrylyl-CoA hydratase; 7, 3-methylglutaconyl-CoA hydratase; 8, 2-mthyl-3-hydroxybutyryl-CoA dehydrogenase; 9, 3-hydroxyisobutyryl-CoA deacylase; 10, 3-hydroxy-3-methylglutaryl-CoA lyase; 11, 2-methyl-acetoacetyl-CoA thiolase; 12, 3-hydroxyisobutyryl-CoA dehydrogenase; 13, methylmalonate semialdehyde dehydrogenase. (Adapted from Valle, 2014).

I.2.1.1 Presentation of organic acidurias

The clinical presentation of the so called “classical” organic acidurias are somehow similar and can be divided into three main forms: i) an acute, severe neonatal onset with metabolic decompensation and neurological symptoms, ii) an acute-intermittent form with recurrent severe metabolic crisis, and iii) a chronic-progressive form presenting with rather unspecific signs like hypotonia, developmental delay and failure to thrive. (Saudybray, 2016)

Patients with the acute neonatal onset form usually have an unremarkable history with a normal pregnancy and delivery at full-term. After a symptom-free period there is a rapid decline of well-being: All OAs except MSUD present with a dramatic clinical picture including dehydration, ketoacidosis, lactate elevation and hyperammonaemia. Hyper- and hypoglycaemia can be found. Blood count alterations (neutropenia, thrombocytopenia, pancytopenia) might be suggestive for a sepsis. Respiratory distress because of ketoacidosis may develop. Patients may present with hypertonic, ophistotonic episodes or axial hypotonia with limb hypertonia and myoclonic jerks. Seizures may develop. Cerebral oedema may lead to a bulging fontanelle and may be misinterpreted as a sign of a neonatal cerebral infection. If treatment is delayed (or too late) children will depict a picture of metabolic encephalopathy progressing to metabolic coma and death. (Baumgartner, 2014)

The acute intermittent form is with 25% less frequent. Patients may develop normally during the first year(s) of live and only present in catabolic states (e.g. infections, fasting, fever) with acute metabolic deterioration. In-between, the episodes the patients may return to normal. Slightly elevated ammonia levels can be found. The development might be normal or delayed with unspecific signs like failure to thrive, recurrent vomiting attacks or episodic, abnormal behavior.

Chronic progressive forms are even less specific in their manifestations. Patients may have a long history of muscular weakness and hypotonia in combination with progressive developmental delay and movements disorders. A history of gastro-intestinal problems including malnutrition and gastro-intestinal reflux may accompany the neurological problems. Classically, patients have an aversion against protein rich food (Saudybray, 2016).

I.2.1.2 Diagnosis of organic acidurias

Although sharing similarities in disease presentation, each OA has its own, metabolic pattern, characterized by the elevation of specific metabolites in patients' body fluids (urine and plasma). These metabolic fingerprints can be detected by the accumulation of characteristic organic acids in patients' urine using gas chromatography mass spectrometry (GC-MS) and by tandem mass spectrometry (MS/MS) which is a complementary technique detecting alterations of the acylcarnitine profile. The latter technique exploits the fact that most acyl-CoA esters are in equilibrium with their corresponding acylcarnitines. A defective single enzyme leads to characteristic elevations of certain products of the acyl-CoA intermediary metabolism (Millington, 2000; Rashed, 1997). Both methods (GC-MS / MS/MS) detect metabolites and intermediary products normally found in healthy individuals but also elevations of metabolites which are usually absent in normal conditions (Chalmers, 1982; Sweetman, 1991). Metabolic deterioration may aggravate the metabolic pattern in the patient while it might be not very pronounced while the patient is well. The diagnosis of organic

acidurias is confirmed by enzymatic assays in patients' cultured cell fibroblasts and finally by molecular genetic analysis.

I.2.1.3 Current opinion on alterations in metabolic flux in organic acidurias

Organic acidurias are caused by monogenic, single enzyme defects leading to an enzymatic block. This leads primarily to the accumulation of specific, potentially toxic products of the intermediary metabolism and to depletion of pathways down-stream the enzymatic block. Mechanistically, this results in impairment of metabolic pathways, activation of by-pass mechanisms, substrate competition for specific transporters and inhibition of enzymes, explaining the clinical and biochemical abnormalities observed in organic acidurias (Fig. 3): In order to compensate for energetic substrates not provided by a blocked gluconeogenic pathway, ketone body formation is up-regulated to fuel beta-oxidation leading to ketoacidosis. Acyl-CoAs are piling up, depleting free Acetyl-CoA in the mitochondria. Accumulating Acyl-CoAs furthermore inhibit the N-Acetyl glutamate synthase, a mitochondrial enzyme catalyzing the first step of the urea cycle essential for the detoxification of ammonia thus leading to hyperammonaemia. Lack of free CoA inhibits the CoA-carnitine exchange mechanism and prevents correct oxidation of substrates from fatty and amino acid oxidation.

As a consequence, disturbances in energy metabolism lead to mitochondrial stress expressed in hyperlactatemia, reduced energy production and oxidative stress (Vockley, 2014).

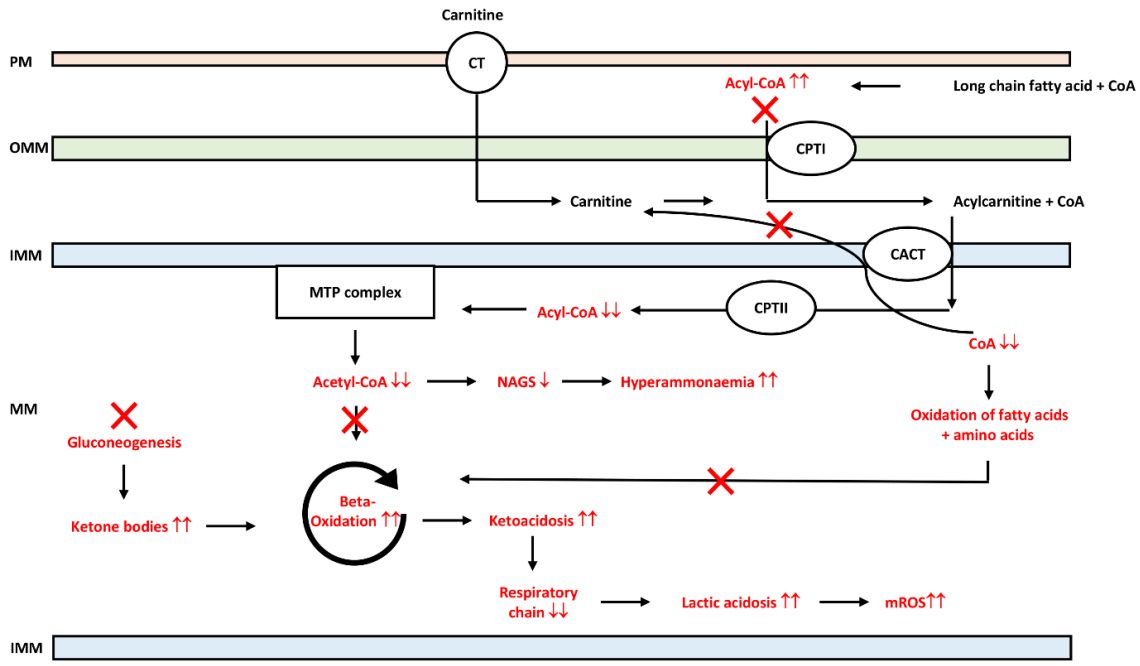

Figure 3: Summary of altered metabolic flux in organic acidurias. *CACT*, carnitine acylcarnitine translocase; *CoA*, coenzyme A; *CPT*, carnitine palmitoyltranferase; *CT*, carnitine transporter; *MTP*, mitochondrial trifunctional protein, *NAGS* N-acetyl-glutamate synthase. *PM*, plasma membrane; *OMM*, outer mitochondrial membrane; *IMM*, inner mitochondrial membrane; *MM*, mitochondrial matrix. Depletion of Acetyl-CoA leads to impaired energy production from beta-oxidation and inhibition of *NAGS* activity resulting in hyperammonaemia. CoA depletion hinders beta-oxidation from fatty acid and amino acid substrates. Consequently, beta-oxidation is fueled by ketone bodies leading to ketoacidosis and respiratory chain dysfunction. (Adapted from: Saudubray, 2016).

I.2.1.4 General treatment principles of organic acidurias

A major therapeutic goal in the management of OAs is to reduce the metabolic flux trough the affected pathways and restore/sustain anabolism: The protein amount is reduced by limitation of natural protein intake and supplementation of amino acid mixtures selectively depleted of BCAAs. A balance between provision of sufficient amounts of protein for development/growth and reduction of metabolic flux via defective pathways has to be found. Furthermore, sufficient energy intake (carbohydrates, fat) is required and has to be increased in situations prone to lead to catabolism (e.g. infections, fever, vaccinations, etc.) which can provoke a live-threatening metabolic crisis. Fasting has to be avoided by all means. Patients' diets are enriched with levocarnitine to support acetyl-CoA formation and facilitate mitochondrial energy metabolism. Oral antibiotics can be used to reduce the propionic acid load produced by gut bacteria. Some enzymes (e.g. propionyl-CoA carboxylase and methylmalonyl-CoA mutase) have an essential co-factor (biotin, adenosylcobalamin (=vitamin B_{12})). In the case of MMA, co-factor responsive patients have been described and administration of hydroxocobalamin (pharmacological form of adenosylcobalmin) improves their metabolic stability and outcome. In acute crisis, scavenger drugs (sodium benzoate, arginine hydrochloride, N-carbamylglutamate) or hemodialysis are needed to counteract hyperammonaemia and prevent cerebral oedema leading to death. In certain organic acidurias, organ transplantation can give metabolic stability (Brassier, 2013; Kashahahara, 2006). There is no causal cure for any of the OAs. Gene or cellular therapy are still pending.

I.2.2 Isolated and combined forms of methylmalonic aciduria

I.2.2.1 Isolated form of MMA

Methylmalonic aciduria (MMA) was first described by the group of Oberholzer (Oberholzer, 1967) in 1967. The prevalence of isolated MMA is about 1:50.000-100.000 (Sniderman 1999; Chace 2001; Shigematsu 2002). Isolated MMA is either caused by mutations of the mitochondrial apoenzyme methylmalonyl-CoA mutase (MUT) encoded by the *MUT* gene (60%), deficiency of the enzyme methylmalonyl-CoA epimerase (*MCEE*, very rare, few cases) or by defects in the processing of 5'-deoxyadenosylcobalamin (AdoCbl), the essential co-factor of MUT (37%). Mutations in AdoCbl processing leading to isolated MMA are due to alterations in the cblA (*MMAA,* 25%), cblB (*MMAB,* 12%) and cblD2 (*MMADHC,* very rare, few cases) complementation group involved in AdoCbl synthesis. The above changes lead to a complete loss (Mut0) or reduced function (Mut-) of MUT activity (Fowler, 2008; Coelho, 2008; Banerjee, 2009) hindering the conversion of D-/L-methylmalonyl-CoA to succinyl-CoA therefore depleting the TCA cycle from anaplerotic substrate (Fig. 4). Biochemically, the disease is characterized by the accumulation of potentially toxic organic

acids (methylmalonic acid, propionate, 3-OH-propionate and 3-methylcitrate) in tissues and body fluids. The most abundant is methylmalonic acid which is pathognomonic and name giving for MMA (Kölker, 2013) (Fig. 4). Elevated propionyl carnitine (C3) levels reflecting the disturbance in acylcarnitine flux are found as well. Disturbed acylcarnitine flux leads to acetyl-CoA depletion of the TCA cycle. The accumulation of diverse monocarboxylic and dicarboxylic short-chain acyl-CoAs (C3 in particular) lead inhibition of the N-acetyl-glutamate-synthase, the first and therefore rate limiting enzyme of the urea cycle (Fig. 4), explaining why secondary hyperammonaemia occurs in MMA patients (Wanders, R, 2014). Hydroxocobalamin responsiveness is frequently found in cblA patients, less frequently in cblB and Mut(-) patients and almost never in Mut(0) patients. It should be carefully evaluated though the prognosis is improved in responsive, treated patients.

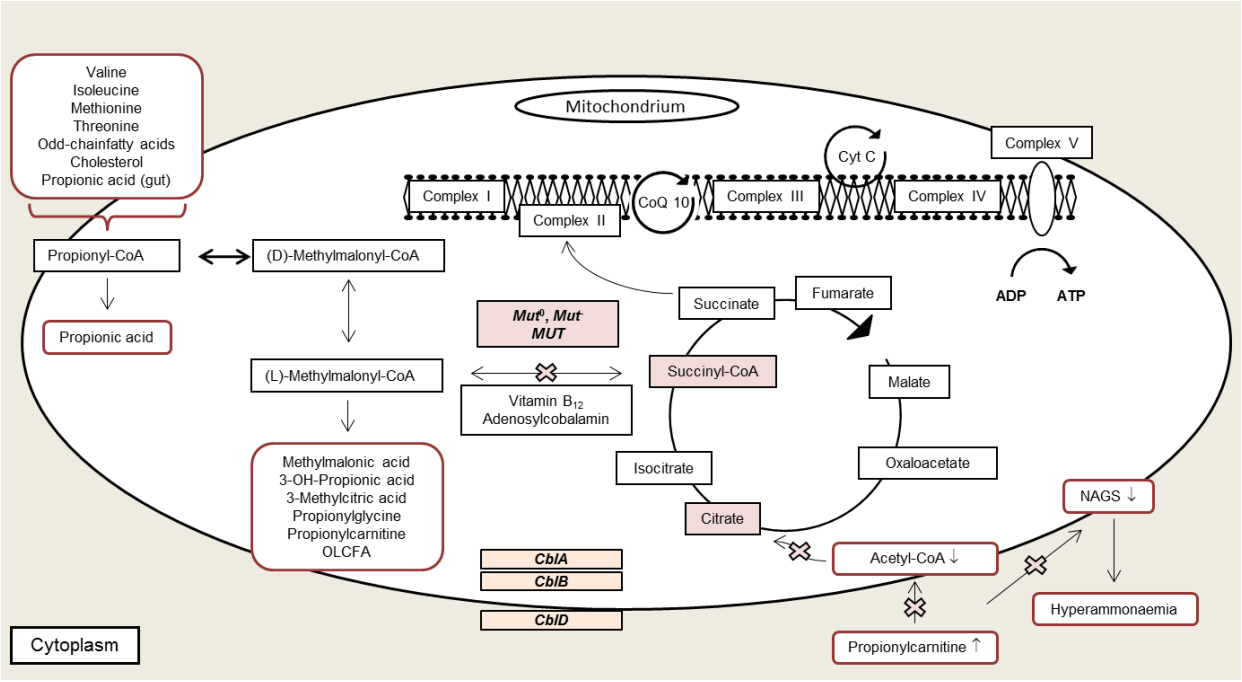

Figure 4: Consequence of mitochondrial MUT deficiency leading to accumulation of specific organic acids, energy depletion of the tricylic acid cycle and hyperammonaemia. (Adapted from Fowler, 2008).

I.2.2.2 Combined forms of MMA

The isolated forms of MMA have to be distinguished from a disease group presenting with combined methylmalonic acidemia and homocystinuria or homocystinuria alone. Isolated and combined MMAs are linked via cobalamin (cbl), an essential cofactor for various reactions. Cbl is taken up by a highly specialized mechanism in the gastrointestinal system and disturbances in the cbl metabolism can affect extra- and intracellular cbl trafficking and processing:

After dietary up-take, cbl is bound to intrinsic factor and absorbed in the ileum via the cubam receptor. Entering the blood stream, cbl is transferred to transcobalamin and taken up by the cell via

endocytosis. Intrinsic factor deficiency (*GIF* gene) and mutations in the subunits of the cubam receptor (*CUBN*, *AMN* gene) as well as transcobalamin deficiency lead to methylmalonic acidemia and homocystinuria caused by disturbance of extracellular trafficking (Allen, 1978 (47); Allen, 1978 (1628); Marcoullis, (1980)). After endocytic up-take in the hepatocyte, cbl is released from transferrin by lysosomal cleavage and exported by the cblJ complementation group with ATP-ase function (*ABCD4*) (Coelho, 2012) and the cblF complementation group (*LMBRD1*) (Rutsch, 2009) in a joined effort (Deme et al, 2014) to the cytoplasm where it undergoes cblC (*MMACHC*) mediated reduction. The processing is continued by the cblD complementation group (*MMACHD*), a multifunctional protein. From this step, 2 pathways diverge: CblD is either processed via mitochondrial synthesis and the cblD2, cblA and cblB complementation group to adenosylcobalamin, the essential cofactor for *MUT* (leading to isolated MMA if steps are deficient), or to methylcobalalmin, the essential cofactor for methionine synthase (MS) and therefore for methylation reactions. Mutations in the cblF, cblJ, cblC and cblD complementation groups lead to combined methylmalonic acidemia and homocystinuria caused by disturbance of intracellular cobalamin trafficking.

Isolated homocystinuria is caused by mutations in the cblD1 complementation group catalyzing the first step of methylcobalamin synthesis needed for MS function. MS, encoded by the *MTR* gene (cblG complementation group), catalyzes the re-methylation of homocysteine to the essential amino acid methionine and plays a major role in the synthesis of creatine, choline and adrenaline as well as in desoxyribonucleic acid (DNA) methylation (Poloscheck, 2005; Hill, 2004). The transfer of methyl-groups is carried out via S-adenosylmethionine (SAM). The product of demethylation is homocysteine again. With time the cob(I)alamin cofactor becomes oxidized to cob(II)alamin rendering MS inactive. Regeneration of MS activity requires methionine synthase reductase (*MTRR*, cblE complementation group) (Zadakova, 2002) in which SAM is used as a methyl-group donor (Leclerc, 1998; Zavadoka, 2002). Deficiencies in CblD1, cblG and cblE complementation groups lead to isolated homocystinuria and low methionine concentrations.

Disturbances affecting homocysteine and methionine metabolism clinically result in bone marrow deficiency, various neurological complications including hypotonia, seizures, severe mental disability and retinopathies.

I.2.2.3 Clinical presentation of isolated MMA

Patients with isolated MMA typically present in the neonatal period after protein intake with hyperammonaemia and severe ketoacidosis leading to an acute, life-threatening metabolic crisis (Ciani, 2000). Moderate lactic acidosis and as well as high glycine levels are frequently found. Neutro- and thrombopenia in combination with vomiting and abdominal distension may lead to confusion with

a sepsis like clinical pattern. Poor weight gain in the postnatal period is usually observed. Acute hemorrhages have been described in a few cases probably due to inappropriate correction of metabolic acidosis. Hypotonia and seizures progressing to lethargy, coma and death evoke if treatment is insufficient or delayed (Saudubray, 2016).

Classical major long-term consequences are “metabolic-stroke”-like episodes causing choreo-athetotic movement disorders and dystonia due to basal ganglia damage (Heidenreich, 1988; Korf, 1986) and tubule-interstitial nephritis (Dudley, 1998; Coman, 2006) leading inevitably to end-stage renal disease. Chronic vomiting and aversion to protein-rich food frequently lead to failure to thrive. Patients may present with severe hypotonia, seizures and movement disorders accompanied by severe intellectual disability. Late-onset optic nerve neuropathy with impaired vision is another insidious complication. Various immunological abnormalities including recurrent infections and pancytopenia have been described. Some patients will present skin disorders resembling acrodermatitis enteropathica-like syndrome (Lane, 2007).

Metabolic crisis can be triggered at any time by catabolism due to e.g. fasting, infections, vaccinations or protein overload. Predictions of the clinical outcome are very difficult to make. There seems to be a correlation between the severity of the mutation and the general disease course. Patients suffering from MMA due to Mut(0) or cblB deficiency have the lowest (or no) MUT activity and the highest MMA levels and are usually more severely affected than Mut(-) or CblA patients. Especially in the kidney disease progression is dependent on the severity of the defect and MMA concentrations (Hörster, 2007). Hydroxocobalamin responsiveness is known to be a protective factor (Haarmann, 2013). For the neurological outcome the frequency of metabolic crisis, early, sufficient management and the damage caused by the previous crises can be used as a prognostic factor (Hörster, 2009).

I.2.2.4 Diagnostic testing in MMA

The clinical pattern described above leads to the suspicion of MMA. Isolated MMA is diagnosed using tandem mass spectrometry (MS/MS) which reveals an abnormal acylcarnitine profile showing elevated levels of propionyl-carnitine (C3). C3 detection alone will not allow the diagnosis of MMA, since it is also elevated in PA due to propionyl-CoA carboxylase deficiency just one step up-stream of MUT. A characteristic pattern for MMA can be identified performing urinary organic acid analysis with gas chromatography–mass spectrometry (GC-MS) which will reveal the pathognomonic metabolite methylmalonic acid as well as 3-methylcitric acid and 3-OH propionic acid besides others. Amino acid chromatography will display non-specific abnormalities such as hyperglycinaemia and hyperalaninaemia. Glutamine levels tend to be low due to metabolic acidosis (Nissim, 1998). The findings need to be further classified either by molecular genetic or enzymatic studies in patients’ skin

fibroblasts in order to group the findings into MUT deficiency or complementation group deficiency by propionate incorporation assay (Willard, 1976) and MUT enzyme assay in the presence or absence of the essential cofactor adenosylcobalamin (Baumgartner, 1983). Complementation analyses are needed for exact assignment to a complementation group (Gravel, 1976). The final diagnosis is confirmed by genetic testing in the proband with subsequent confirmation of the mutation in the parents.

I.2.2.5 Treatment options in isolated MMA

Treatment principles in MMA have to be divided into acute and chronic interventions.

Acute metabolic crisis requires the complete stop of protein-intake for maximal 24 (-48h). High dosage of i.v. glucose if necessary in combination with insulin is required to induce anabolism. Application of i.v. lipids can be used to support anabolism. Hyperammonaemia is a potentially life-threatening complication accounting for a high percentage of mental disability in the patients and ammonia levels have to be reduced as fast and effective as possible. This can be achieved by the use of scavenger drugs (sodium benzoate, L-arginine hydrochloride) and N-carbamylglutamate antagonizing propionyl-CoA induced hyperammonaemia. Hyerammonaemia is most efficiently reduced by hemodialysis, -filtration or peritoneal dialysis. However, in certain circumstances due to circulation issues in babies and little experience for this age group, novel therapies using peritoneal dialysis and small nanoparticles highly specific binding ammonia without causing peritoneal damage might soon become novel therapeutic options (Devuyst, 2015).

The principles of long-term treatment are sufficient calorie intake to avoid catabolism and a protein restricted diet especially limited for the intake of the precursor amino acids which cannot be broken down. Carnitine is supplemented at 100 mg/kg/d to fuel mitochondrial energy metabolism. Co-factor responsive patients will profit from hydroxocobalamin supplementation.

These measures, however, do not prevent metabolic derailments and long-term complications. Chronic kidney disease is inevitably diagnosed in every MMA patient earlier or later in dependency of the severity of the mutation and methylmalonic acid concentrations. Hemo- and peritoneal dialysis are needed to bridge the time until kidney transplantation.

Since liver and kidney are the organs with the highest MUT expression (Wilkemeyer, 1993) and people running on 20% residual enzyme activity can lead a normal live, it was believed that organ transplantation might cure MMA. However, it turned out that transplantation of either combined liver and kidney or liver and kidney alone gives increased metabolic stability in many cases, but still does not prevent patients from metabolic stroke like events and long-term complications (Brassier, 2006; Kasahara, 2013). Therefore, new treatment options are needed to slow down or even stop disease

progression. To gain new insights into disease mechanism, cellular models to investigate subcellular effects and animal models to investigate whole organ(ism) effects of the disease are required.

I.2.2.6 Pathophysiology of kidney disease in MMA

Patients suffering from MMA, even when mildly affected, are known to be at risk of developing CDK. The severity of the mutation is directly related to the concentration of accumulating methylmalonic acid, a well-known risk factor for onset and progression of CKD (Hörster, 2007).

MUT is expressed in all human tissues, high concentrations are found in the kidney, where renal epithelial cells depent on mitochondrial energy production for various important transport processes (Emma, 2016). Renal tubular dysfunction and tubulointerstitial lesions are frequently found in MMA patients (Baumgartner, 2014; Hörster, 2007). The cellular mechanisms linking MUT deficiency and renal epithelial dysfunction have not been elucidated to date. Several mechanisms have been proposed to explain the pathophysiology. First, disturbances of the TCA cycle and the respiratory chain as well as alterations in the transport of the anti-oxidant glutathione have been thought to be involved in cellular damage (Morath, 2008). As a consequence of mitochondrial dysfunction oxidative stress and mitochondrial DNA depletion might lead to disturbed cellular homeostasis and function (De Keyzer, 2009; Kölker, 2006; 2013). Second, accumulating organic acids (methylmalonic acid in particular) might exert toxic effects on cellular function. According to the "trapping-hypothesis", impaired organic acid transport across the blood-brain barrier as well as *de novo* synthesis has been associated to brain damage in MMA (Kölker, 2006; 2013). Sauer et al. (2009) suspect a similar pathomechanism for mitochondrial dysfunction in the kidney. Finally, the detection of tubulo-interstitial nephritis in kidney biopsies of MMA patients led to the suspicion of an underlying inflammatory stimulus (Lubrano, 2007; Ruppert, 2015).

The generation of *Mut* knock-out mouse models was hampered by the fact of neonatal lethality (Peters H, 2008). Following mouse models were dependent on AAV-based gene therapy (Chandler, 2010) or transgenic expression ($Mut^{-/-}$; $Tg^{INS\text{-}Alb\text{-}Mut}$) for survival (Manoli, 2013). Protein load was able to induce a renal phenotype in $Mut^{-/-}$; $Tg^{INS\text{-}Alb\text{-}Mut}$ mice with reduced glomerular filtration rate, tubulointerstitial damage and increased renal damage markers. Aditionally, abnormal mitochondrial morphology and function was detected. The use of a constitutive *Mut* knock-in allele derived from a patient's mutation recently allowed the generation of mouse models recapitulating phenotypic traits with a genetic-dosage effect presenting with progressive CKD and oxidative stress over time (Forny, 2016).

However, it remains unclear, how MUT deficiency influences mitochondrial integrity and homeostasis and how mitochondrial fate is linked to renal epithelial cell damage.

I.3. Impact of mitochondrial function on human health and disease

I.3.1 Mitochondrial energy metabolism

Mitochondria, cytoplasmic organelles derived from the engulfment of an eukaryotic progenitor by an alpha-proteobacterium are the only organelles to have their own genome (mtDNA), which is organized in a single double-stranded loop, lacking introns and using a different genetic code as compared to nuclear encoded genes. MtDNA codes for 37 genes and includes 13 subunits of respiratory chain proteins. The inheritance is exclusively maternal. More than 6% of the active genome contributes to function and homeostasis of mitochondria emphasizing their key role for the organism (Suomalainen, 2015).

Based on their specific structure with strictly separated outer (OMM), inter (IMS) and inner (IMM) mitochondrial membrane spaces, mitochondria are enabled to generate adenosine triphosphate (ATP) via oxidative phosphorylation along the respiratory chain. The respiratory chain consists of 5 enzymatic complexes (complex I-V) and 2 electron carriers (CoQ_{10}, cytochrome c) residing along the christae of the IMM (Nunnari, 2012). Acetyl-CoA derived from the breakdown of carbohydrate and lipid metabolism enters the TCA cycle producing NADH and $FADH_2$ which are reduced by complex I and complex II. The electrons are transported via electron transporters and complex III to complex IV, where they reduce O_2 to H_2O. The energy produced is used to pump protons across the IMM leading to an electrochemical gradient needed by complex V for ATP production (Fig. 5). Other important tasks in cellular metabolism include the production of redox substrates, regulation of cell signaling and apoptosis as well as biosynthetic functions. This sum of tasks makes mitochondria important sensors of cellular metabolic stress and allows for cellular adaptation to environmental factors (Vyas, 2016).

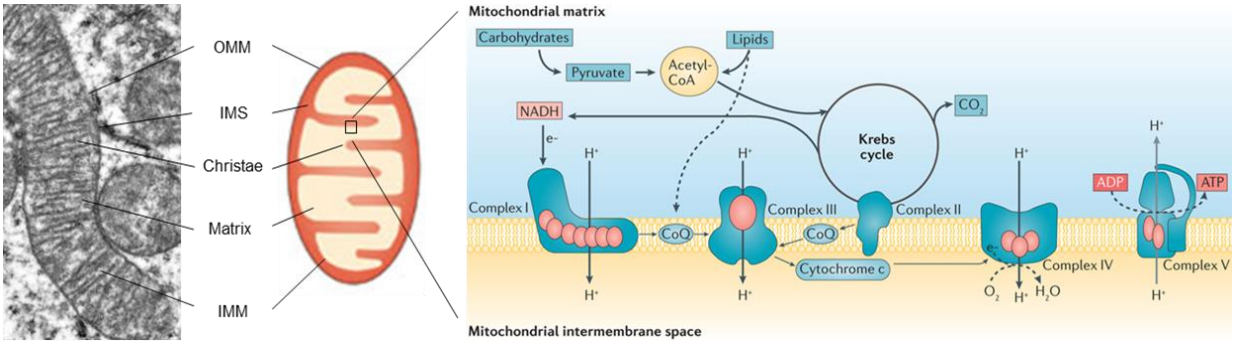

Figure 5: Mitochondrial morphology and structure allows a unique form of energy production via the respiratory chain. Acetyl-CoA is an important product of carbohydrate and lipid metabolism. ATP is produced via oxygene reduction along the highly specialized respiratory chain. (OMM=outer mitochondrial membrane, IMS=intermembrane space, IMM=inner mitochondrial membrane, CoQ=Coenzyme Q10). (Adapted from Emma F, 2016, Sheng ZH, 2012).

I.3.1.1 Disorders in mitochondrial energy metabolism

Disorders of mitochondrial energy metabolism caused by mutations in mitochondrial and/or nuclear DNA are a genetically highly heterogeneous group of different diseases (Suomalainen, 2015). They display both tissue specific manifestations and clinical diversity (Nunnari, 2012) and may present at any age. The overall prevalence of mitochondrial disorders is probably greater than 1:5000.

Primary mitochondrial disorder syndromes frequently present with optic nerve atrophy, sensorineural deafness, cardiomyopathy, muscular weakness and encephalopathy and many show various degrees of renal tubular dysfunction. Renal Fanconi syndrome is frequently present in patients with Pearson syndrome (Niaudet, 1994) or patients with CoQ_{10} deficiency (Niaudet, 1997; Emma 2012). Only a few primary mitochondrial diseases present with isolated symptoms (e.g. Leber hereditary optic nerve neuropathy) causing isolated blindness. Secondary (acquired) mitochondrial dysfunction is caused by many different hereditary diseases leading to impairment of mitochondrial function without affecting genes encoding for structural or functional mitochondrial proteins (Nunnari, 2012). Acquired mitochondrial dysfunction has been described e.g. in kidney disease. Studies provide evidence for increased ROS levels in common causes for CKD like diabetes mellitus, arterial hypertension and glomerulonephritis leading to initiation and aggravation of kidney damage (Che, 2013).

Mitochondrial well-being is considered essential for tissue functionality and excessively elevated ROS levels have been identified as a major disease-driving burden. However, recent studies provide evidence ROS signaling is a selective, dosage-dependent and target-specific process (Schieber, 2014) playing an important role in cell signaling, proliferation and cell survival. Mitochondrial ROS signaling is an essential second messenger for an efficient immune system (West, 2011) (Kamminski, 2013). Inflammation on the contrary, is associated with high ROS levels, resulting in tissue damage. This highlights the narrow border between physiological and pathophysiological effects of ROS and explains why mitochondria, important sources of ROS themselves, need a dynamic defense to maintain integrity and homeostasis.

I.3.1.2 Mitochondrial dynamics and homoeostasis

Mitochondria play a fundamental role in regulating cellular metabolism. Tight control of mitochondrial functions and dynamics is crucial to maintain an adequate energy balance. Adaption to different stressors (ROS) is critical for the maintenance of mitochondrial integrity and homeostasis.

Functionally, the underlying process refers to mitochondrial fission and fusion, a process in which morphological changes of the mitochondrial network allow the separation of dysfunctional from healthy mitochondria. The process is mediated by Mitofusin 1 and 2 (Mfn1, Mfn2) required for outer membrane fusion, while inner membrane fusion is promoted by the dynamin like protein encoded by

OPA1. Fission is tightly regulated by Dynamin related protein 1 (Drp1). Disturbances in these delicate, well balanced processes have been associated with mitochondrial diseases (Chen H, 2009).

In dependency on the extent of ROS induced damage several lines of defense have been developed. Mitochondria have an intrinsic proteolytic system to eliminate misfolded proteins (Matsushima 2012) (Fig. 6a). Dysfunctional outer mitochondrial membrane proteins can be removed by proteosomal degradation (Karbowski, 2011) (Fig. 6b). Furthermore, oxidized products can "bud" of mitochondria for lysosomal degradation (McLelland, 2014), (Fig. 6c). The diversity of this disposal pathway underlines the importance of mitochondrial integrity for energy supply and highlights ROS as a major disease-causing burden.

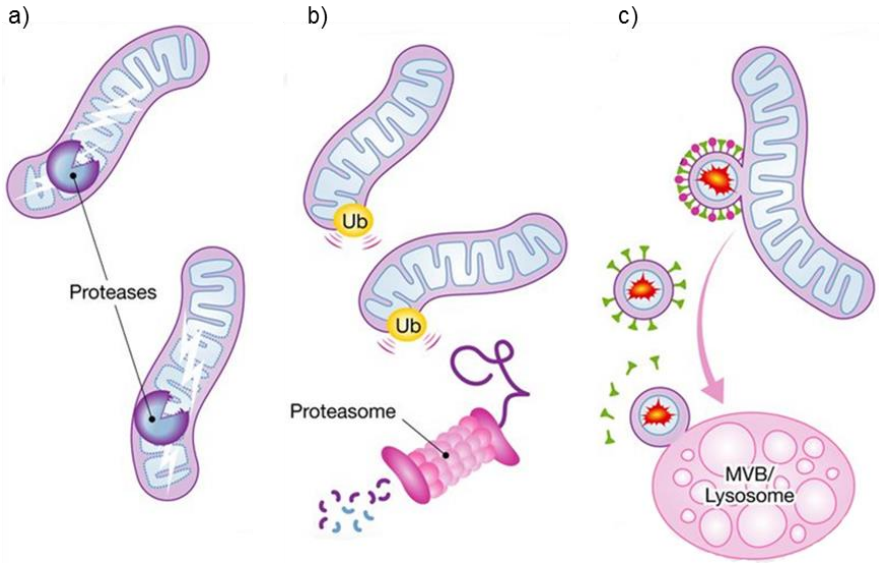

Figure 6: Mitochondrial quality control. In dependency of the severity of damage mitochondria can use different strategies to protect themselves from ROS. a) Degradation of misfolded proteins by proteases of the intermembrane space or mitochondrial matrix; b) Degradation of misfolded protein on the outer mitochondrial membrane by proteasomal degradation or c) budding of outer mitochondrial membrane compounds for lysosomal degradation. (Adapted from Sugiura, 2014).

Mitochondrial degradation is counter acted by mitochondrial biogenesis under the control of numerous nuclear transcription factors and co-activators (Liang H, 2006). The most extensively studied is peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1-α. PGC-1-α is modulated by extracellular signals controlling metabolism, cellular growth and differentiation (Scarpulla, 2011) as well as by the important intracellular nutrient and energy sensors adenosine monophosphate-activated protein kinase (AMPK) and sirtuin1 (SIRT1) (Scarpulla, 2011). Highly expressed in metabolically active organs, PGC-1-α expression can be linked to the mitochondrial distribution pattern in the kidney with highest levels in the renal cortex and tubular epithelial cells and lowest in the glomerulus (Tran, 2007). In acute kidney injury, PGC-1-α expression is down regulated followed by return to normal levels when organ function recovers (Tran, 2007).

Taken together, these findings point at a complex interplay of mitochondrial separation and degradation on the one hand and biogenesis on the other hand rendering possible mitochondrial homeostasis and integrity. Both systems are tightly connected to cellular metabolic needs and impairment will lead to severe disturbance of cellular energy metabolism.

I.3.1.3 Mitochondrial dysfunction in renal disease

Different studies have pointed at the special relationship between mitochondrial energy production (generation of ATP), mitochondrial well-being and kidney function (Verdin 2015; Tran, 2016). Although the kidneys only account for 0.5% of body mass, they consume 10% of the total oxygen production (Berg 2002). Especially the proximal tubule (PT) and the thick ascending limb (TAL) of the kidney are rich in mitochondria and therefore vulnerable to mitochondrial dysfunction. This is due to active reabsorption and excretion processes against concentration gradients at high energy costs: The proximal tubule of the kidney is involved in specialized transport processes that are critical to maintain homeostasis, reabsorbing two thirds of filtered solutes and water. The energy driving these processes is mainly generated by the basolateral Na^+/K^+-ATP-ase. Various co- and counter transporters are dependent on gradients produced by active, ATP-consuming, transport mechanisms. Multi-ligand receptors lining PT-cells are responsible for the endocytic up-take of various ligands. Further processing along the endolysosmal pathway requires vesicular acidification by ν-ATP-ase before re-distribution of the recycled substances can occur (Prange, 2016). In the thick ascending limb urine is concentrated again requiring active transport processes by Na^+/K^+-ATP-ases. Electrolytes (Ca^{2+}, Mg^{2+}) are reabsorbed by Ca^{2+}/Mg^{2+}- sensing receptors (Ferre, 2012). Mitochondria are increasingly recognized to be the key organelles for proper kidney function and mitochondrial dysfunction plays a leading role in the pathophysiology of kidney disease (Emma, 2016).

Unsurprisingly, many mitochondrial disorders are characterized by various degrees of renal tubular dysfunction. Complete Fanconi syndrome, presenting with low-molecular weight proteinuria and glucose loss represents global dysfunction of PT cells (Emma, 2012). Partial defects including renal tubular acidosis, isolated glycosuria or aminoaciduria are frequently observed. Patients presenting with a Bartter-like phenotype and hypermagnesuria pointing at involvement of the distal nephron and the TAL have also been reported (Emma, 2011; 2006). Mitochondrial impairment in podocytes leads to disruption of the filtration barrier and protein loss. Two major entities have been identified: Glomerulopathies due to defects in the CoQ_{10} biosynthesis, an important shuttle of electrons and anti-oxidants, and due to the mtDNA mutation 3243 A>G in the $tRNA^{Leu(UUR)}$ gene (Emma, 2016).

In most cases, mitochondrial damage is acquired. As renal tubules are highly active in terms of transport and metabolism, acute kidney injury (of toxic, septic or ischemic etiology) causes early

changes the epithelial-lining mitochondrial population. Excessive ROS production leads to morphological and functional changes. Loss of the characteristic christae structure, mitochondrial swelling, disruption and loss of mitochondrial mass have been observed predominantly in the PT but also in the TAL and the distal tubule (Brooks, 2009; Manny, 1980). These defects in mitochondria of the renal tubular epithelium underline the fact, that high energy consumption and vulnerability are closely related and that mitochondrial dysfunction is accompanied by ultrastructural damage.

Common diseases like diabetes mellitus and arterial hypertension are known to induce chronic kidney injury. Studies have shown increased ROS production and inactivation of complex IV pointing at mitochondrial dysfunction in these patients. (Che, 2013). It appears that there is a correlation between degradation of renal function and increasing ROS levels. ROS in turn is believed to stimulate CKD progression (Cachofeiro, 2008). In this context, different mediators and markers of inflammation (interleukin-6, tumor necrosis factor-α, cyclooxygenase I) were found to be elevated suggesting an inflammatory process which is triggered by ROS. Persistent inflammation might be another factor leading to progression of CKD (Landray, 2004).

The current opinion on kidney injury caused by mitochondrial dysfunction states, that impairment of mitochondrial function not only leads to decreased ATP but also increased ROS production (Fig. 7). ROS released from damaged kidney cells furthermore amplifies ROS levels. Structural mitochondrial alterations lead to release a of mtDNA working as a pro-inflammatory signal and cytochrome c, a trigger for apoptosis (Zhang, 2010), thereby possibly driving renal epithelial dysfunction and CKD. Tran et al. recently showed that substitution with nicotinamide adenine dinucleotide, an important player of the respiratory chain, improves kidney function after ischemic insult, providing evidence that control of ROS levels and mitochondrial health is not only important for the maintenance of kidney function but also plays an important role in tissue repair (Tran, 2016).

In the last years, mitochondria have been identified as the key organelles for onset and progression of kidney damage and ROS has been identified to be a major disease driving burden. The use of mitochondria targeted anti-oxidants has been shown to have beneficial effects by ROS reduction and improvement of kidney damage (Szeto, 2011). Targeting pathways which effect mitochondrial dynamics, mitophagy and biogenesis might offer other, novel approaches to ameliorate progression of kidney damage and sustain kidney function.

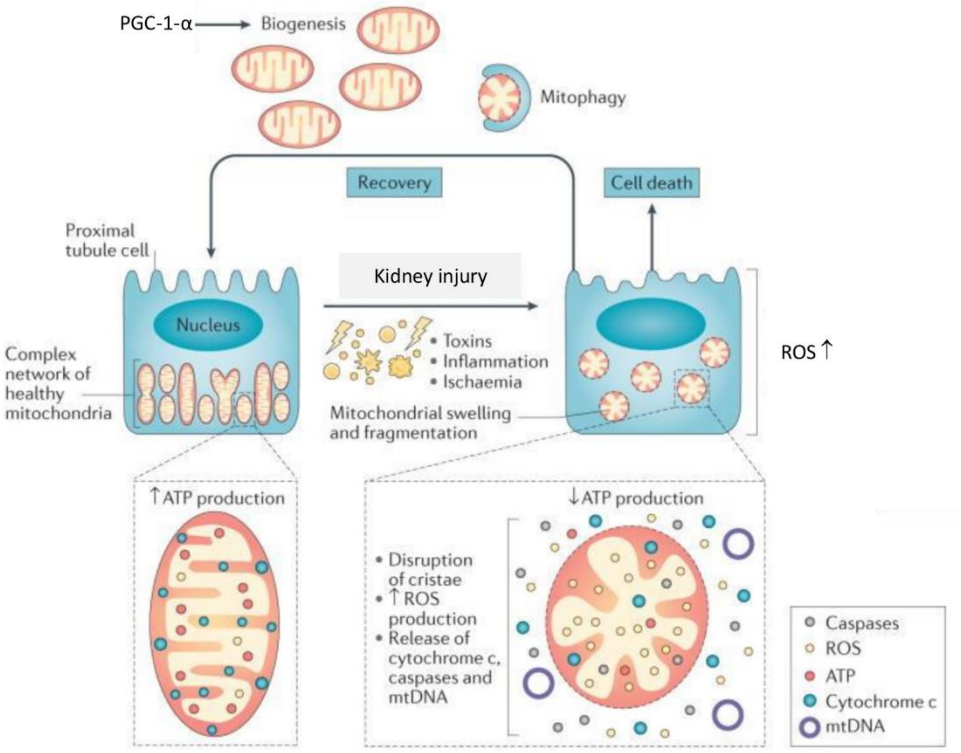

Figure 7: Mitochondrial injury and recovery in renal epithelial cells. Renal epithelial cells are highly active in terms of transport processes and rich in mitochondria providing ATP. Multiple stimuli induce mitochondrial injury leading to organellar swelling, fragmentation and dysfunction. Dysfunctional mitochondria release a cocktail of harmful molecules such as ROS and cytochrome c potentiating mitochondrial injury and promoting cell-death if uncontrolled. Recent studies suggest that clearance of dysfunctional mitochondria by mitophagy/autophagy and reconstitution of mitochondrial mass by mitochondrial biogenesis, a process mediated via proliferator-activated receptor-γ co-activator 1-α (PGC-1-α), are involved in the recovery process. (Adapted from Emma F, 2016).

I.3.2 Autophagy and mitophagy

Mitochondria are essential organelles that regulate cellular homeostasis and concomitantly tissue function. The removal of dysfunctional mitochondria is critical to maintain proper cellular function. Elimination of defective mitochondria either occurs via autophagy, a non-selective cellular recycling process or via mitophagy, an organelle-specific break-down pathway. Both pathways are essential for mitochondrial quality control (MQC).

Autophagy, also known as self-digestion, is a crucial cellular degradation process. Its main function is to provide metabolites in times of shortage and to serve as quality control by clearing misfolded proteins and dysfunctional organelles. Recent evidence suggests that autophagy can not only be induced by starvation but also in nutrient-rich conditions pointing at a key role for autophagy in catabolic and anabolic metabolism (Kaur, 2015).

Defects in the autophagic pathway result in the accumulation of dysfunctional products (e.g. organelles, proteins) and have been associated with severe infectious, malignant, renal, immunological, cardiovascular and neurodegenerative chronic diseases (Sureshbabu, 2015).

Autophagy is a tightly orchestrated multi-step process starting with the formation of an isolation membrane (phagophore), elongation of this membrane and cargo recruitment, formation of a double-layer membrane (autophagosome), fusion with the lysosome (autolysosome) and finally degradation (Fig. 8).

Dysfunctional cargo gets ubiquitinated (e.g. by ubiquitin ligase) which allows selective recognition of dysfunctional products by the autophagic receptor p62/SQSTM1. p62/SQSTM1 labelled cargo attracts microtubule associated protein light chain 3 (LC3) for autophagosome formation (Lenoir, 2016). Autolysosome formation is mediated by RAB7, an important regulator of late endocytic membrane trafficking. After complete fusion of the autolysosome and the lysosome the cargo is degraded by different enzymes in the acidic lysosomal environment. Nutrients are recycled and transported into the cytoplasm (Nixon, 2013).

Autophagy itself is tightly controlled by the mammalian target of rapamycin complex (mTORC1), adenosine monophosphate-activated protein kinase (AMPK) and sirtuin1 (SIRT1), important sensors for nutrient state, oxygen consumption and energy status of a cell. Activation of mTORC1 leads to inhibition of autophagy. AMPK and SIRT1 on the other hand, are autophagy inducers (Lenoir, 2016) as is the catabolic state induced by starvation.

While degradation via autophagy is rather unspecific, mitophagy exclusively removes damaged mitochondria via this organelle specific pathway (Fig. 8). The process is initiated after separation by mitochondrial fission. Major players are the mitochondrial serine/threonine-protein kinase PINK1 and the E3 ubiquitin ligase parkin. PINK1 is rapidly turned over by proteolysis in healthy mitochondria. During mitochondrial dysfunction PINK1 is no longer cleaved from the OMM thereby attracting parkin. Parkin recruitment leads to ubiquitination of OMM structures. The ubiquitin binding-adaptor p62/SQSTM1 binds to parkin-ubiquitinated mitochondria mediating the binding of LC3 which allows degradation via the lysosomal pathway (Youle, 2011).

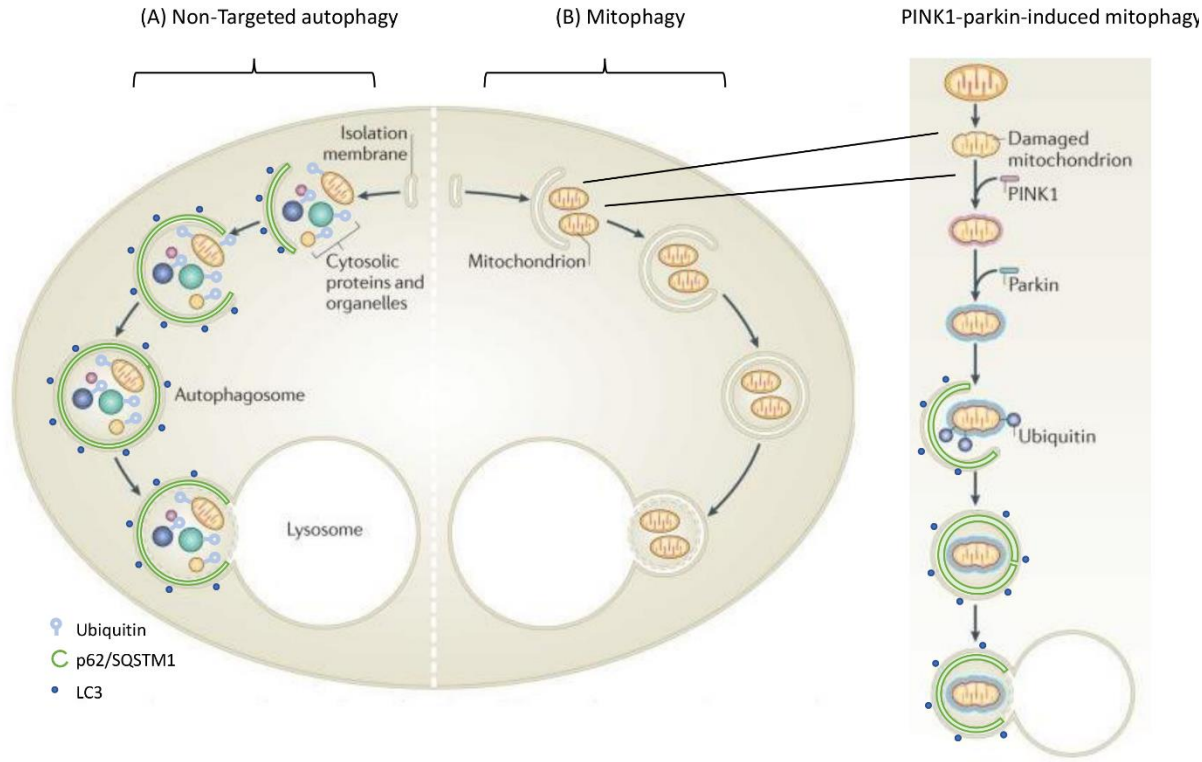

Figure 8: Mitochondrial quality control. **(A)** Non-selective autophagy degrades cytosolic compounds and different organelles. After ubiquitination, recruitment of p62/SQSTM1 and LC3 induce autophago-lysosomal fusion and lysosomal digestion. **(B)** Mitophagy selectively eliminates defective mitochondria. Dysfunctional mitochondria express PINK1 on the OMM attracting parkin. Parkin-ubiquitinated mitochondria attract the autophagic machinery for lysosomal degradation. (Adapted from Youle, 2011).

It is generally accepted, that ROS induces autophagy and activation of autophagy counteracts ROS (Scherz-Shouval, 2011) in return. Mitochondrial dysfunction, ROS and activation of autophagy are therefore tightly related. Disturbance in this key system for cellular homoeostasis can be monitored e.g. by studies of autophagic flux, trafficking of cargo between different compartments or by measurement of lysosomal enzymatic activity. P62/SQSTM1 or LC3 are often used to monitor autophagic processes. It has to be kept in mind, that accumulation of autophagic proteins can be induced by any step down-stream in the autophagic process and either due to increased autophagic flux, inhibition of autophogo-lysosomal fusion as well as inhibition of lysosomal function. To correctly evaluate the status of autophagic flux, co-localization studies of different subcellular compartments to visualize correct targeting, inhibition of certain processes (e.g. lysosomal degradation with Bafilomycin) to reveal changes in autophagosome synthesis and investigation of lysosomal dynamics (content of lysosomal proteins, activity of lysosomal enzymes) have to be performed (Klionsky, 2016; Mizushima, 2004).

I.3.2.1 The role of autophagy in kidney disease

Autophagy is a tightly controlled lysosomal degradation pathway removing damaged organelles and aggregated proteins to preserve cellular homeostasis and integrity. Defective autophagic clearance is involved in the pathogenesis of a variety of systemic and metabolic diseases. Autophagy plays a crucial role in metabolically active, mitochondria-rich organs like the kidney (Lenoir, 2016). Recent studies link renal cellular damage in the tubulo-interstitial and glomerular compartment (Fig. 9) to dysregulated autophagic processes (Ding, 2015; Lee, 2014; Fougeray, 2015; Liu, 2014).

Renal epithelial cells are rich in mitochondria to perform active transport processes and are extremely vulnerable to ROS. Recent studies suggest that autophagy is important to preserve mitochondrial integrity and homeostasis in proximal (Namba, 2014; Kimura, 2011) and distal tubular cells (Pallet, 2008). Disturbance of the autophagic process induced by genetic manipulation resulted in elevated ROS levels, accumulation of autophagic proteins and kidney damage.

The importance of autophagic clearance has also been demonstrated in different models for acute kidney injury like ischemia-reperfusion (Jiang, 2010) and cisplatin treatment (Periyasamy-Thandavan, 2008). Acute damage led to autophagy induction while inhibition of the process worsened tissue damage. Chronic metabolic acidosis is a severe complication of CKD and frequently found in patients with organic acidurias leading to mitochondrial stress and dysfunction. Namba et al. (Namba, 2014) recently provided evidence, that metabolic acidosis induces autophagy in renal tubule cells and that activation of autophagy is crucial for the maintenance of mitochondrial homeostasis in these conditions. Activation of the autophagic pathway is also observed in other chronic (Lenoir, 2015; Lim, SW) kidney disease conditions and believed to be renal-protective.

Interestingly, disturbances of the autophagic pathway have been linked to progressive renal fibrosis. In several mouse models for fibrosis induction, autophagic markers were increased. Pharmacological inhibition of autophagy worsened tubule-interstitial fibrosis progression (Kim, 2012; Ding, 2014). In line with that, autophagy was found to foster degradation of transforming growth factor beta (TGF-β), which is known to promote fibrosis progression and aggravate kidney disease (Ding, 2014).

The role of autophagy in the glomerular compartment has been extensively studied in diabetes mellitus characterized by causing progressive podocyte destruction over time (Najafian, 2011). Robust evidence suggests, that high glucose levels lead to ROS production followed by activation of the autophagy machinery in podocytes. High glucose concentrations furthermore induce autophagy directly (Lenoir, 2016). Long term exposure to high glucose levels induces a down-regulation of the autophagy machinery which seems to sustain podocyte damage (Fang, 2013). The group of Gödel et al (Gödel, 2011) provides evidence, that this is due to mTORC1 activation, which might provide some

short-term benefits but ultimately leads to glomerulosclerosis and podocyte destruction. These data sustain the renal-protective effect of autophagy induction for the glomerular compartment.

It is commonly believed, that autophagy has a reno-protective role and that impairment of the autophagy pathway leads to kidney disease progression. The mechanism by which autophagy protects renal tubular cells is currently unclear (Huber, 2012). Novel insights from this thesis suggest, that inter-connected mechanisms like general and organelle specific degradation of dysfunctional organelles might be part of that complex pathway. Further investigations are needed to unravel the precise role of autophagy in different disease entities and cell types. A comprehensive understanding of the underlying network will facilitate the discovery of novel therapeutics to prevent CKD.

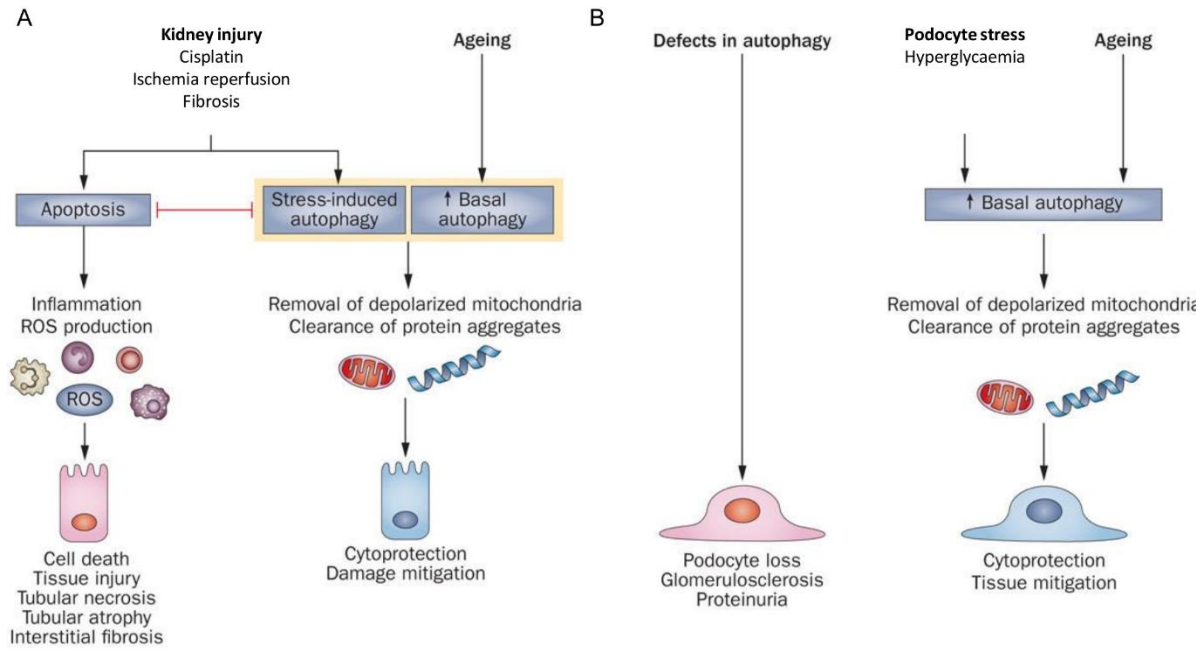

Figure 9: Putative cyto-protective functions of autophagy in tubular cells and podocytes **(A)** Protection of renal tubular cells from acute and chronic damage by clearance of defective mitochondria, protein aggregates and ROS reduction. **(B)** Defects in the autophagic pathway induce podocyte loss and glomerular damage. Activation of autophagy removes defective mitochondria and protein clusters restoring podocyte function. (Adapted from Pallet, 2015).

II. AIMS OF THE THESIS

The PhD Thesis consist of 2 main parts aiming to shed new light on how the deficiency of the monogenic enzyme MUT leads to systemic and subcellular changes driving onset and progression of CKD in MMA in a multilevel approach.

1I.1 Characterization of the classical disease course and possible influence of adenosylcobalamin (vitamin B_{12}) on the progression of CKD in Methylmalonic aciduria (MMA)

The first section deals with the long-term follow up of a patient suffering from a vitamin B_{12} responsive CblA type MMA-uria due to the relatively common p.R145X STOP mutation. Despite metabolic stability and normal development and growth, the patient developed chronic kidney disease (CKD) at the age of 12 years. Of note, kidney function could be stabilized by the administration of vitamin B_{12} for a period of 13 years before the patient had to undergo renal replacement therapy.

This case report illustrates:

i) The long-term follow-up of a patient with MMA-uria due to cblA deficiency;

ii) The involvement of the kidney as a target organ despite a relatively mild metabolic phenotype;

iii) The importance of early and adequate vitamin B_{12} substitution in responsive patients;

II.2 Mechanistic studies in the different mouse and human models reveal mitochondrial dysfunction and impaired autophagy to drive kidney damage in Methylmalonic aciduria

The MUT enzyme is ubiquitously expressed in tissues of mouse and humans. However, long-term consequences in MMA predominantly affect the brain (basal ganglia damage) and the kidney (CKD), which are highly energy dependent organs and therefore rich in mitochondria. For this reason, the mitochondrion has been suspected to be the key organelle related to CKD in MMA.

We used a multi-level approach in human derived samples (tissue, renal epithelial cell lines) and different animal models ($Mut^{ki/ko}$ mouse model, $Mut^{flox/flox}$ mouse model) to:

i) Characterize MUT expression in mouse tissues and human renal cells;

ii) Investigate mitochondrial morphology and function in MMA;

iii) Evaluate the role of the autophagy and mitophagy in MMA;

iv) Recapitulate findings in the novel MMA mouse model ($Mut^{ki/ko}$);

v) Exploit Cre mediated *Mut* deletion in mPTC to investigate the causal role of MUT;

vi) Investigate the possible impact of anti-oxidants in human MMA cells

III. Renal involvement in a patient with cobalamin A type (cblA) methylmalonic aciduria: a 42-year follow-up

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Abstract

Chronic renal failure is a well-known long-term complication of methylmalonic aciduria (MMA-uria), occurring even under apparently optimal metabolic management. The onset of renal dysfunction seems to be dependent on the type of defect and vitamin B_{12}-responsiveness. We report on a patient with a vitamin B_{12}-responsive cobalamin A type (cblA) MMA-uria caused by a homozygous stop mutation (p.R145X) in the cobalamin A gene *(MMAA)*. She was diagnosed with chronic kidney disease (CKD) stage III at the age of 12 years. Following re-evaluation, the patient received vitamin B_{12} (hydroxocobalamin) treatment, resulting in a significant decrease in the concentration of methylmalonic acid (MMA) in urine and plasma. Until age 29 years glomerular filtration rate remained stable probably due to hydroxocobalamin treatment slowing down progression to end-stage renal failure. Kidney biopsies showed non-specific manifestations of chronic interstitial inflammation. The patient received a renal transplant at age 35 years. Under continuous treatment with hydroxocobalamin there is no evidence of kidney damage due to MMA-uria until the last follow-up 6 years after transplantation.

This case report illustrates (i) a long-term follow-up of a patient with MMA-uria due to cblA deficiency, (ii) the involvement of the kidney as a target organ and (iii) the importance of early and adequate vitamin B_{12} substitution in responsive patients. Further investigation will be necessary to prove the protective effect of hydroxocobalamin on the kidney in vitamin B_{12}-responsive patients.

Abbreviations

AdoCbl	adenosylcobalamin
cblA	methylmalonic aciduria cblA type OMIM 251100
cblB	methylmalonic aciduria cblB type OMIM 251110
cblD-variant 2	methylmalonic aciduria and homocystinuria cblD type OMIM 277410
CKD	chronic kidney disease
eGFR	estimated GFR
MMA	methylmalonic acid
MMA-uria	methylmalonic aciduria
MC	2-methylcitric acid
MUT	methylmalonyl-CoAmutase OMIM 609058
mut^-	partial defect of methylmalonyl-CoA mutase activity OMIM 251000
mut^0	complete defect of methylmalonyl-CoA mutase activity OMIM 251000
3-OH-PA	3-OH-propionic acid
PA	propionic acid

Keywords

Methylmalonic aciduria, vitamin B_{12} responsiveness, chronic kidney disease, kidney biopsy, end-stage renal disease, kidney transplantation

1. Introduction

Methylmalonic aciduria (MMA-uria), first described by the group of Oberholzer et al. in 1967 (1), belongs to the group of organic acidurias. It is biochemically characterized by the accumulation of specific organic acids such as methylmalonic acid (MMA), propionate (PA), 3-OH-propionic acid (3-OH-PA) and 2-methylcitric acid (MC). The disease is caused either by mutations (mut^0, mut^-) in the enzyme methylmalonyl-CoA mutase (MUT) which is involved in the break-down of branched chain amino acids, odd-numbered chain fatty acids, cholesterol side chains and other metabolites or by mutations in the genes *MMAA* (cblA), *MMAB* (cblB) and *MMADHC* (cblD-variant2) encoding proteins important for the synthesis of its cofactor adenosylcobalamin (AdoCbl) (2). According to the type of mutation and resulting residual enzymatic activity, there is a wide clinical spectrum of the disease. Typically, patients manifest with acute metabolic crisis during the first days of life or in catabolic states due to infectious diseases or prolonged fasting. The metabolic crises are characterized by lactic acidosis, hyperketonaemia, hypo- or hyperglycaemia and hyperammonaemia and may result in metabolic encephalopathy and multi-organ failure. Complications include failure to thrive, developmental delay, mental retardation, muscular hypotonia, metabolic stroke like episodes with basal ganglia damage and renal failure (3).

The pathophysiology of chronic kidney disease (CKD) in MMA-uria is poorly understood. There is evidence that the severity of the defect and therefore the concentration of MMA might play an important role (3). The nature of the disease in the kidney is unclear for the lack of specific biomarkers and investigations of kidney biopsies at an early stage of the disease. Careful clinical descriptions combined with detailed studies of kidney biopsies are necessary to yield insights into the origin of kidney destruction. Here we present the 42-year history of renal involvement as well as analysis of several kidney biopsies in a female patient with vitamin B_{12}-responsive MMA-uria due to a homozygous stop mutation in the *MMAA* gene (cblA).

2. Case report

The patient was born after uneventful pregnancy as the first child to a non-consanguineous Caucasian couple. She presented at 3 days of life with icterus (bilirubin 340 µmol/l), muscular hypotonia, vomiting, weight loss and lethargy. There was no acidosis present (plasma bicarbonate 25.4 mmol/l) and blood ammonia concentration was normal for a newborn (88 µmol/l). The first profile of amino acids in plasma and urine revealed elevated concentrations of lysine and hyperlysinemia was ruled out. Under suspicion of a metabolic defect the child was put on a low protein diet (1.4 g protein/kg/d) and improved remarkably. The follow-up investigation of the amino acid profile showed hyperglycinemia and hyperglycinuria. Accidental protein intake of 2 g/kg/d at 4 months of age led to a metabolic derailment which could be easily controlled by i.v. glucose application. Aged 8 month the urine was screened for organic acids and high concentrations of MMA were detected in urine (4.3 mmol/d) and plasma (255 µmol/l) establishing the diagnosis of MMA-uria. Incorporation of label from (^{14}C)propionate into cell proteins in cultured fibroblasts was severely deficient but showed a clear response to supplementation of the culture medium with high concentrations of hydroxocobalamin. A cblA defect was confirmed by somatic complementation and later by molecular genetic investigation which revealed a homozygous mutation in exon 2 of the *MMAA* gene (c.433C>T) predicting an amino acid change from arginine to a stop codon at position 145 (p.R145X). Vitamin B_{12} concentration was low (225 pmol/l) but within the normal range (220-730 pmol/l). Intramuscular injections of hydroxocobalamin for 5 days did not change the concentration of excreted MMA. Accordingly, no treatment was started initially. The patient was kept on a low protein diet (1.4 g/kg/d) and showed normal growth and psychomotor development (IQ 98 at age 4 years by J. Kramer intelligence test) (Fig. 1).

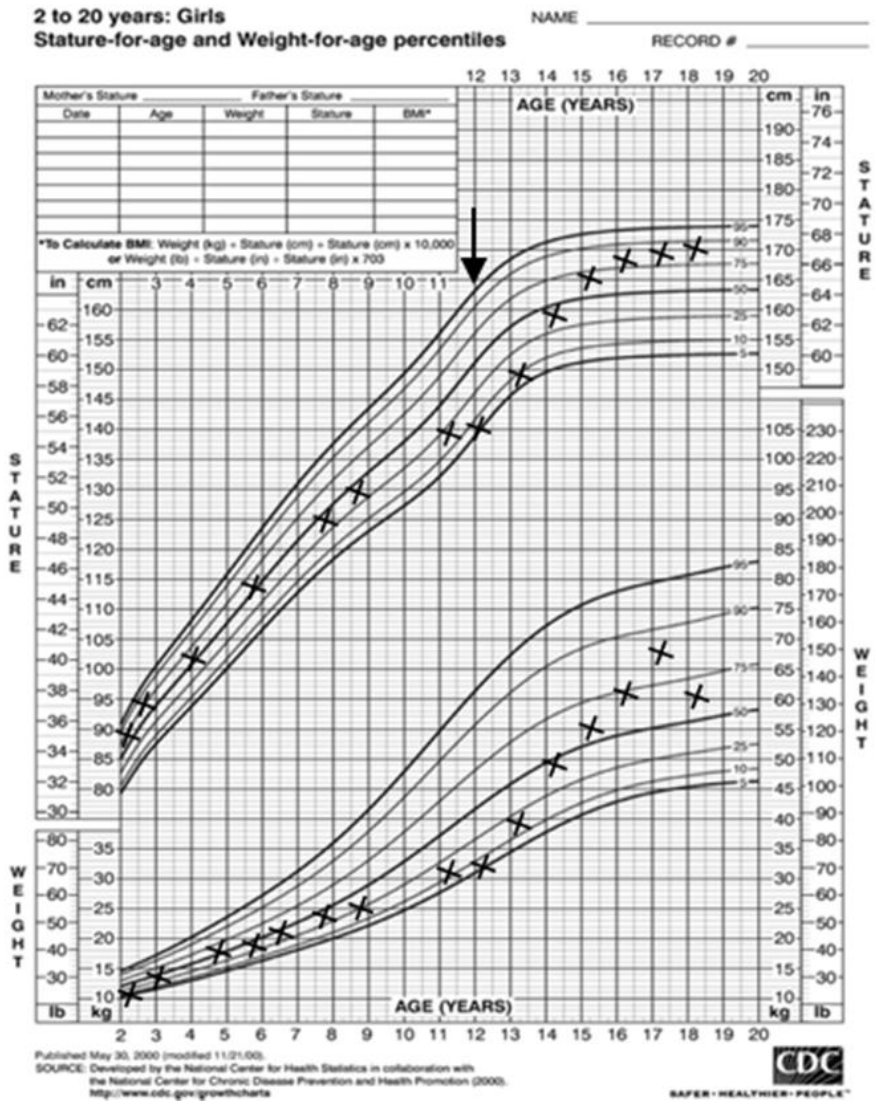

Figure 1: Summary of physical growth. The percentiles show stature and weight-for-age. CKD was diagnosed because of a decline in growth at 12 years of age as indicated by arrowhead.

At age 12 years, the patient showed impaired growth and CKD stage III was discovered (glomerular filtration rate: 45 ml/min/1.73 m^2, obtained by 24h urine collection, (Fig. 2)). Until this age, kidney function had not been documented so that the onset of CKD remains obscure. In addition, hyperuricemia (up to 778 µmol/l) due to reduced excretion of uric acid (19 µmol/kg/d, normal range 91±50) was identified and subsequently treated with a xanthine oxidase inhibitor (Allopurinol®). Generalized signs of proximal tubulopathy, like that seen in Fanconi syndrome described by D'Angio et al. in a patient with MMA-uria (4) were not found. Kidney size determined by ultrasound was within the normal range. According to a previous publication (5) the responsiveness of this patient to hydroxocobalamin was re-evaluated and, this time, found to be positive. Treatment with

hydroxocobalamin was re-introduced using a dosage of 2x10 mg/d p.o. from 1983-1995 and 1x10 mg/7d i.m. from 1995 to 2006. Cofactor responsiveness was documented by impressive catch-up growth (Fig. 1) as well as long-term decline in urinary MMA excretion (Fig. 2), with mean MMA excretion decreasing from 3212 µmol/mmol creatinine before administration to 719 µmol/mmol creatinine (oral treatment) and further to 422 µmol/mmol creatinine (i.m. administration). Treatment included a low protein diet as well as carnitine (3x1 g/d) and bicarbonate. Neurodevelopment remained normal (IQ 97 at age 22, by Hamburg-Wechsler-Intelligenztest für Erwachsene, (HAWIE-R)).

Total kidney volume detected by ultrasound decreased during the following years and at 16 years of age it was below two standard deviations compared to healthy peers. From 1988 she had arterial hypertension and in 1989 (age 18), at CKD stage III (GFR 54 ml/min/m^2, determined by inulin clearance), a renal biopsy was performed (Fig. 2, nr. 1, Fig. 3). The main findings were areas with advanced tubular atrophy and interstitial fibrosis which were interspersed with mononuclear infiltrates. Immunohistochemistry and electron microscopy (EM) ruled out a glomerulonephritis or a hereditary glomerulopathy. Unfortunately, the material was not sufficient to examine the tubulointerstitium by EM in respect to mitochondrial pathologies as seen in the proximal tubules of a *Mut* knock-out mouse model (6).

From 1996 (age 25), renal function deteriorated. Another biopsy was performed in 1999 (age 28), (Fig. 2, nr. 2) showing a similar pattern as previously reported. However, additionally severe arteriolosclerosis was detected most probably due to arterial hypertension. One year later renal replacement therapy had to be started and the patient was dependent on hemodialysis the following 6 years (Fig. 2).

In 2006 (age35) the patient underwent kidney transplantation from a deceased donor. After the transplantation she remained on a low protein diet as well as supplementation with hydroxocobalamin (1x4 mg/14d i.m.) and carnitine (3x1 g/d). Immunosuppressive regimen consisted of tacrolimus (Prograf®, Astellas), mycophenolate-sodium (Myfortic®, Novartis) and prednisone. Prednisone was tapered and stopped three months after transplantation. The estimated glomerular filtration rate

(eGFR) according to Cockroft Gault was 90 ml/min/1.73 m^2. Plasma MMA concentration decreased from 304 µmol/l to a baseline of 59 µmol/l (Fig. 2). Graft function remained stable and surveillance biopsies (Fig. 2, nr. 3, 4) were performed according to the transplant protocol three and six month after transplantation revealing borderline and moderate tubulitis, respectively, both consistent with interstitial rejection. The latter was treated with steroid pulses. In the second year after transplantation (eGFR 68 ml/min/1.73 m^2) a third renal biopsy (Fig. 2, nr. 5) was performed. Besides minor arteriolosclerosis the morphology was well preserved and no further abnormalities were reported. Blood pressure was well controlled under antihypertensive treatment. At the last follow-up, six years after transplantation, serum creatinine concentration was 95 □mol/l, the eGFR was 70 ml/min/1.73 m^2 and there was neither glomerular nor tubular proteinuria detectable.

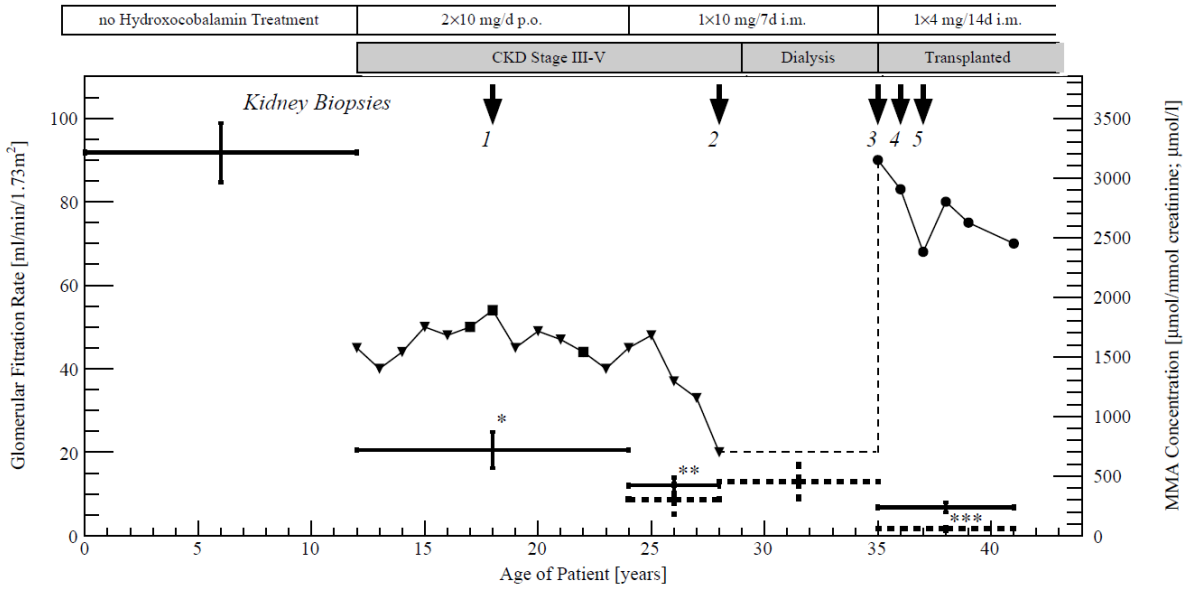

Figure 2: Course of glomerular filtration rate (GFR) and concentration of MMA in an individual with cblA MMA-uria in dependence of hydroxocobalamin (OHCbl) treatment. Values for GFR indicated as triangles were obtained as mean, each single value obtained from a 24 h urine collection (n=5 (1983), n=6 (1984), n=8 (1985), n=5 (1986), n=5 (1987), n=8 (1990), n=7 (1991), n=3 (1992), n=4 (1994), n=10 (1995), n=5 (1996), n=7 (1997), n=4 (1998), n=3 (1999)). GFR values indicated as squares were measured by inulin clearance, values indicated as circles were obtained by Cockroft Gault estimation. Urinary MMA values (µmol/mmol creatinine) are depicted as a black line and plasma MMA (µmol/l) as a dotted line. Error bars are represented by vertical lines. Kidney biopsies are indicated with arrowheads and numbered. Boxes show 4 periods of treatment with hydroxocobalamin (n = number of independently performed measurements): No hydroxocobalamin treatment (n=47), 2x10 mg/d p.o. (n=21), 1x10 mg/7d i.m. (urine: n=42, plasma: n=6), post transplantation 1x4 mg/14d i.m. (urine: n=32, plasma: n=28). The significance has been determined by one-way ANOVA (*, ***P* <0.05) for MMA concentration in urine and (****P* <0.05) for MMA concentration in plasma and is referring to the previous value.

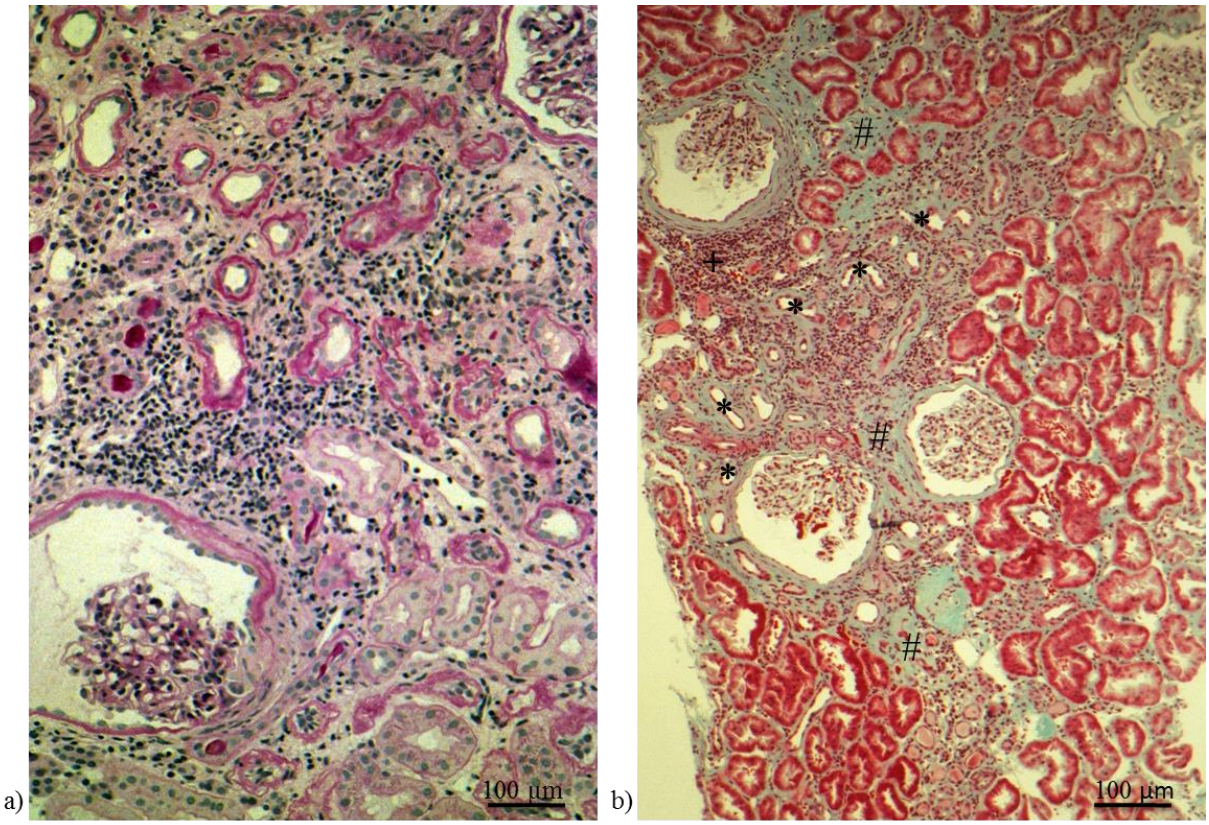

Figure 3: Native kidney biopsy (1989, age 18 years) showing dense inflammatory interstitial infiltrate (+), areas of tubular atrophy (∗) and interstitial fibrosis (#). Enrichment of protein casts close to the medulla-cortex region was reported as well as large areas with completely inconspicuously looking tubules (data not shown in the pictures). a) Periodic acid Schiff stain (PAS), LM, 200x; b) Masson's Trichrome Stain (TRI), LM, 200x.

3. Discussion

We present a long-term follow-up of a patient with vitamin B_{12}-responsive cblA type MMA-uria due to the STOP mutation p.R145X, which is the most common allele found in patients with CblA defects (representing 43% of pathogenic alleles) (7). The case illustrates progressive loss of kidney function finally leading to end stage kidney failure in a patient with a rather mild form of MMA-uria, in whom development and growth are normal and severe metabolic decompensations are lacking. It also underscores the importance of standardized testing for cofactor responsiveness and subsequent treatment with hydroxocobalamin in such patients. Our data clearly suggest improved metabolic control on i.m. application *vs.* oral supplementation. Given the risk of these patients for severe long-term complications (e.g. end stage renal disease and optic nerve atrophy) it is important to study the effects of more aggressive management (8).

Patients with isolated MMA-uria, even mildly affected, are thought to be at risk of developing CKD (3). High concentrations of MMA in urine are a known risk factor for the development of CKD in those patients (3). The administration of vitamin B_{12} in our patient resulted in a significant decrease in urinary MMA excretion (Fig. 2). Partial correction of the biochemical phenotype has slowed the progression from CKD III to end stage renal disease. Only after 17 years of vitamin B_{12} supplementation, the patient necessitated hemodialysis. Nevertheless, these results should be interpreted with caution, since the urinary excretion of MMA and other putatively toxic metabolites of alternative propionate oxidation (e.g. 2-methylcitrate) diminish with decreasing kidney function and thus accumulating toxic compounds could foster the naturally occurring disease progression despite hydroxocobalamin supplementation. Unfortunately, only very few plasma MMA levels are available during the period before end stage renal disease. The drop of mean plasma as well as urinary MMA concentrations after renal transplantation (Fig. 2) is most likely due to the transplant, as the kidney is said to contribute to at least 18% of activity normally provided by the liver (9, 10).

Several mechanisms may explain the pathophysiology of cellular damage in MMA. First, disturbances of the tricarboxylic acid cycle and the respiratory chain as well as disturbances in the glutathione and dicarboxylate transport are thought to be involved in the pathomechanism (11).

Secondary to mitochondrial dysfunction, oxidative stress and disturbances in mitochondrial DNA equilibrium might occur (12, 13, 14). Second, accumulating dicarboxylic acids may be toxic, in particular in the brain. According to the "trapping hypothesis" impaired transport of dicarboxylic acids either at the blood-brain barrier or between astrocytes and neurons as well as de novo synthesis of dicarboxylic acids in brain is causing brain damage (13, 14). Sauer et al. (11, 15) suspect a similar pathomechanism of impaired function of dicarboxylic acid transporters and accumulating mitochondrial toxins to be responsible for kidney damage in MMA-uria.

The performed native kidney biopsies showed areas with remarkable tubular atrophy and interstitial fibrosis interspersed with mononuclear infiltrates without other abnormal findings which might reflect the influence of MMA-uria in the kidney (Fig. 3). These observations fit with the description of kidney biopsies taken from other patients with MMA-uria due to mut^- and cblA deficiency (10, 16). Similarly, the graft biopsies did not show any striking abnormalities which might be a hint at kidney damage caused by MMA-uria. This finding matches with the group of Lubrano et al. (17) who reported the case of a girl receiving a kidney transplant at the age of 17 years because of MMA-uria due to a cblA defect. In a 16.5 year follow-up study renal function remained stable and re-biopsy did not show any striking abnormalities, even during pregnancy (18). In contrast to our case, this patient is on unrestricted diet and has not been on hydroxocobalamin treatment (18).

Because of the small number of organ transplantations in a phenotypically highly variable patients' population a clear recommendation for organ transplantation in MMA-urias cannot be made. Thirty-one patients with MMA-uria with kidney (n=10), liver (n=15) or combined liver/kidney transplantation (n=6) have been reported so far (10, 16, 20, 21, 22, 23, 24) most of them harboring a mut^0 (n=16) defect. Some patients underwent episodes of severe metabolic decompensation, metabolic stroke or death (25) even after combined liver and kidney transplantation clearly demonstrating that severe complications may still occur. Nevertheless, the kidney seems to play an important role for metabolic stability and correction of MUT activity in the patients as kidney or combined kidney/liver transplantation seem to lower MMA concentration more efficiently and provide more metabolic stability than single liver transplantation (10, 17, 24, 26). This case report as well as the findings of

other groups (17, 24) in even more severe forms of MMA-uria suggests that elective kidney transplantation may be a form of “cell-therapy” and regarded as an alternative and safer strategy than liver or combined liver-kidney transplantation for it seems to restore sufficient enzyme activity and improves quality of life in patients.

Several cases of hyperuricemia in MMA-uria have been reported (10, 27) similar to the finding in our patient. As suggested by the reduced excretion of uric acid, one could speculate that the characteristically accumulating organic acids -and MMA in particular- may induce tubular damage and inhibit the tubular secretion of uric acid. Uric acid is known to induce gout nephropathy via chronic interstitial inflammation (28) and might be an additional toxic factor in the development of CKD in MMA-uria.

In conclusion, this case report illustrates that CKD is a long-term complication even in patients with mild defects leading to almost no metabolic derailment. High concentrations of MMA are suspected to be nephrotoxic and hydroxocobalamin can lower MMA concentration in responsive patients. Responsiveness to hydroxocobalamin treatment should therefore be carefully evaluated and responsive patients should be adequately treated by parental administration since this may slow down the progress of CKD. Elective kidney transplantation seems to be a valid option in selected patients. To date, no evidence of recurrence of MMA-specific changes in the transplanted kidney has been evidenced; however, it has to be kept in mind that it is neither curative nor protective against metabolic stroke.

Conflict of interest

The authors disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years after submission that could inappropriately have influenced, or be perceived to influence, their work.

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IV. Impaired Mitophagy Links Mitochondrial Dysfunction and Epithelial Cell Damage in Methylmalonic Aciduria

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Abstract

The maintenance of mitochondrial integrity has a key role in preserving both the metabolic and cellular homeostasis in specialized cells. Mitochondrial disorders such as methylmalonic aciduria (MMA), due to inactivating mutations in the *MUT* gene coding for methylmalonyl-CoA mutase (MUT) cause epithelial cell damage resulting in renal tubular dysfunction and kidney disease. The mechanisms linking mitochondrial defects and epithelial cell damage remain unknown, preventing the development of disease-modifying therapies.

By combining genetic and pharmacological approaches, we demonstrate that mitochondria are fragmented and dysfunctional in human and mouse renal cells lacking functional MUT. Through the dysregulation of the PINK1/Parkin pathway, disease-causing *MUT* mutations or *Mut* gene deficiency impair the autophagy-mediated clearance of dysfunctional mitochondria. In turn, the cells accumulate autophagy and ubiquitinated proteins and mitochondrial ROS, causing cell damage. Treatment of MMA patient-derived cells with the mitochondria-targeted antioxidant MitoTEMPO (MT) restored mitochondrial homeostasis and increased the ratio of uncleaved vs. cleaved PINK1, promoting autophagy degradation of dysfunctional mitochondria and reversing the cellular damage process. These data reveal the mechanism bridging mitochondrial dysfunction and epithelial cell damage in MMA and the importance of PINK1/Parkin dependent mitochondrial turnover in epithelial cell homeostasis. Targeting mitochondrial ROS production offers therapeutic perspectives to reduce epithelial damage downstream to primary mitochondrial defects.

1. Introduction

The mitochondrial network performs diverse yet interconnected functions, producing energy and many biosynthetic intermediates while also contributing to the maintenance of homeostatic signaling pathways (Nunnari, 2012). The functioning of this dynamic and pleiotropic organelle is particularly relevant in the context of terminally differentiated cells (McWilliams, 2017). Defects in mitochondrial function potentially confer devastating vulnerability to many different cell types, contributing to severe clinical features of a wide range of human disorders (Anding, 2017).

Methylmalonyl-CoA mutase (MUT) is a mitochondrial enzyme which catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA using vitamin B_{12} as a cofactor. This reaction is crucial for the propionate catabolism, funneling metabolites of odd-chain fatty acids, branched-chain amino acids and cholesterol into the Krebs cycle (Baumgartner, 2014). The vital importance of MUT activity is demonstrated by methylmalonic aciduria (MMA, MIM# 251000), a severe inborn error of metabolism that is primarily caused by recessive mutations in the encoding *MUT* gene, resulting in complete (mut^0) or partial (mut^-) deficiency of the enzyme. Patients with MMA usually present during the first days of life with severe metabolic acidosis, lethargy, and failure to thrive, progressing to multi-systemic complications, coma and even death when untreated (Oberholzer, 1967; Baumgartner, 2014). MUT is highly expressed in the kidney, where mitochondria fuel the important transport processes mediated by renal tubular cells (Emma, 2016). The most common complications observed in surviving MMA patients include renal tubular dysfunction and development of chronic kidney disease due to tubulointerstitial lesions (Hörster, 2007; Cosson 2009; Baumgartner, 2014). Treatment for MMA is limited to the reduction of circulating metabolites, symptomatic care of complications, and liver and/or kidney transplantation (Brassier, 2013; Kassahara, 2006). Thus, there is an urgent need to identify novel therapeutic targets for this devastating disorder.

Despite the genetic elucidation of MMA and the increasing knowledge of its clinical characteristics, the pathophysiology of the end-organ damage and the cellular mechanisms linking mitochondrial enzyme deficiency and epithelial dysfunction in MMA remain largely unknown. The *Mut* knock-out (KO) mouse models needed AAV-based gene therapy (Chandler, 2010) or transgenic expression in the liver (Manoli, 2013) to overcome the neonatal lethality, limiting their relevance for

the study of the human disease (Forny, 2016). Studies in transgenic *Mut*$^{-/-}$; Tg$^{\text{INS-Alb-}Mut}$ mice showing a decreased glomerular filtration rate with tubulointerstitial damage revealed abnormal mitochondria, oxidative stress and increased renal damage markers, potentially attenuated by antioxidants (Manoli, 2013). The generation of more clinically relevant mouse models of MMA using a constitutive *Mut* knock-in (KI) allele based on a typical MMA patient mutation (p.met700Lys) allowed us to confirm the metabolic relevance of the model, with manifestations of progressive kidney damage and markers of oxidative stress (Forny, 2016).

In most mammalian cells, a variety of mitochondrial quality control (MQC) systems operate to preserve the mitochondrial architecture and its bioenergetic function (Fischer, 2012). Through an evolutionary conserved, selective degradation of worn-out/damaged mitochondria by autophagy (a process called mitophagy), the cells remove the potential damage-promoting byproducts, safeguarding the homeostasis and cellular functions (Stolz, 2014). During mitophagy, the kinase PINK1 and the ubiquitin ligase Parkin cooperate to attach ubiquitin tags to proteins of outer membrane of damaged mitochondria, which mark them for disposal (Allyson, 2017; Kitada, 1998). Autophagy receptor proteins such as SQSTM1/p62 then assist in cellular degradation by binding to both the ubiquitinated proteins and to components of autophagy machinery, such as the protein LC3 (Stolz, 2014). This homeostatic process is particularly active in renal tubular cells, whose reabsorptive and secretory activities require the maintenance of mitochondria network (Fougeray, 2015). Moreover, the autophagy-mediated turnover of damaged mitochondria is required for protecting the tubular cells from acute injury (Isaka, 2011), whereas deletion of essential autophagy genes damages them through defective mitochondrial clearance and increased reactive oxygen species (ROS) (Yamamoto, 2016). Of note, accumulation of abnormal mitochondria with disorganized cristae has been described in kidney biopsies from MMA patients (Manoli, 2013), suggesting potential involvement of autophagic-mediated clearance and MQC systems.

We therefore hypothesized that the MUT enzymatic deficienty may alter the integrity of the mitochondrial network by compromising mitochondrial autophagy and that such defects may contribute to the renal tubular dysfunction observed in patients with MMA. Here, we report that disease relevant inactivating mutations or genetic deletion of MUT impair the PINK/Parkin-mediated

autophagic degradation of fragmented and dysfunctional mitochondria, causing oxidative stress and dysfunction of renal tubule cells. Restoring mitochondrial homeostasis by applying mitochondria-targeted antioxidant treatment in MMA patient-derived cells reverse cellular dysfunction, emphasizing a new therapeutic strategy for treating renal tubular dysfunction in MMA and mitochondrial disorders.

2. Results

Defective mitochondrial homoeostasis, oxidative stress and damage in tubular cells from MMA patients

Based on the high expression and specific distribution pattern of MUT in mouse and human kidney (Suppl. Figs. S1 and S2), we hypothesized that the MMA-associated mutations inactivating MUT impair mitochondria homeostasis and promote tubular/epithelial cell dysfunction.

To tackle this hypothesis, we first analyzed the mitochondrial network in renal tubular cells obtained from the urine of MMA patients harbouring *MUT* mutations (MMA1-3) and three controls (WT1-3) (Table 1). The MMA-derived renal tubular cells displayed a marked decrease in MUT protein and activity at TOMM20-labelled mitochondria when compared to control cells (Fig. 1, A-C).

Transmission electron microscopy (TEM) analyses revealed that mitochondria, which appear as an interconnected meshwork of elongated organelles in control cells, were characterized by a prominent fragmented/rod-like shape with an impaired cristae organization in MMA cells (Fig. 1D). The outer mitochondrial membrane protein TOMM20 and inner mitochondrial membrane protein prohibitin showed a marked decrease in mitochondrial content in MMA cells (Fig. 1E). These changes contrasted with no changes in segment markers (Suppl. Fig. S3A-B), morphological features (Suppl. Fig. S3C), viability (Suppl. Fig. S3D) and cell proliferation rates (Suppl. Fig. S3E) in MMA cells compared to controls.

We next tested whether these MUT-induced changes in mitochondria network could have any impact on organelle function. Measurement of overall oxygen consumption rates in control and MMA patient-derived cells based on Seahorse metabolic flux analyses revealed impaired mitochondrial bioenergetics, as evidenced by a reduction in baseline respiration, ATP turnover and total respiratory capacity (Fig. 1F; Suppl. Fig. S4 A-B). Quantitative confocal imaging of tetramethylrhodamine methyl ester (TMRM) dye revealed that the resting mitochondrial membrane potential ($\Delta\psi_m$, a key requisite for effective oxidative phosphorylation and energy production) of MMA cells was significantly lower than of control cells (Fig. 1G). These changes were paralleled by major mitochondrial oxidative stress, as indicated by elevated mtROS levels (MitoSOX, a live-cell permeant indicator of mitochondrial superoxide; Fig. 1H), increased anti-oxidant response (SOD1; Fig. 1I), and tubular cell damage,

indicated by the major increase in the kidney injury molecule 1 (KIM1) and lipocalin-2 (LCN2) (Fig. 1I) in the MMA cells. These data suggest that an abnormal mitochondrial network triggers oxidative stress and epithelial damage in MMA patient-derived renal tubular cells.

Table 1. Mutations and enzymatic activities in renal tubular cells obtained from wild-type controls and patients with MMA.

Source	Class of MUT mutation	Nucleotide change	Amino acid change	Enzymatic activity (pmol/min/mg protein)
Control 1	WT	-	-	3403 ± 136
Control 2	WT	-	-	7406 ± 336
Control 3	WT	-	-	3738 ± 218
MMA1	mut^-	c.572C>A (exon 3)	p.A191E	79 ± 9
MMA2	mut^0	c.862T>C (exon 4)	p.S288P	17 ± 10
MMA3	mut^0	c.982C>T (exon 4)	p.L328F	38 ± 25

Table 1: Enzymatic activity is given as mean ± SEM of 3 independent measurements. WT, wild-type; MMA, methylmalonic aciduria; mut^0, complete deficiency; mut^- partial deficiency in methylmalonyl-CoA mutase (MUT).

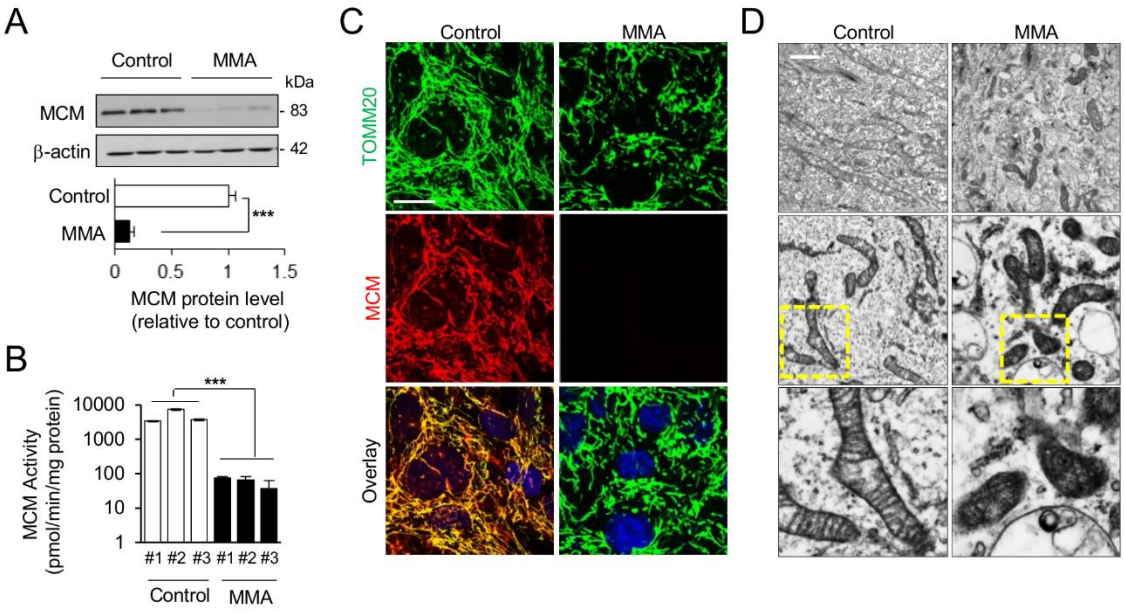

Figure 1. Impaired mitochondrial homeostasis, oxidative stress and cell damage in tubular cells derived from MMA patients.

(A) Western blotting and densitometric analyses of MUT protein levels in control (n=3) and MMA cells (n=3). β-actin was used as a loading control. ***P<0.001 relative to control cells. (**B**) MUT enzymatic activity in crude cell homogenates obtained from control (n=3) and MMA cells (n=3). ***P<0.001 relative to control cells. (**C**) The control and MMA-derived renal tubular cells were immunostained with anti-TOMM20 (green) and anti-MUT (red) antibodies, and analysed by confocal microscopy. The MMA cells show a marked decrease in MUT protein and activity at TOMM20-labelled mitochondria when compared to control cells. (**D**) Electron microscopy showing filamentous mitochondria with preserved cristae in control cells vs. fragmented mitochondria with destroyed cristae in MMA cells. Dotted yellow squares.

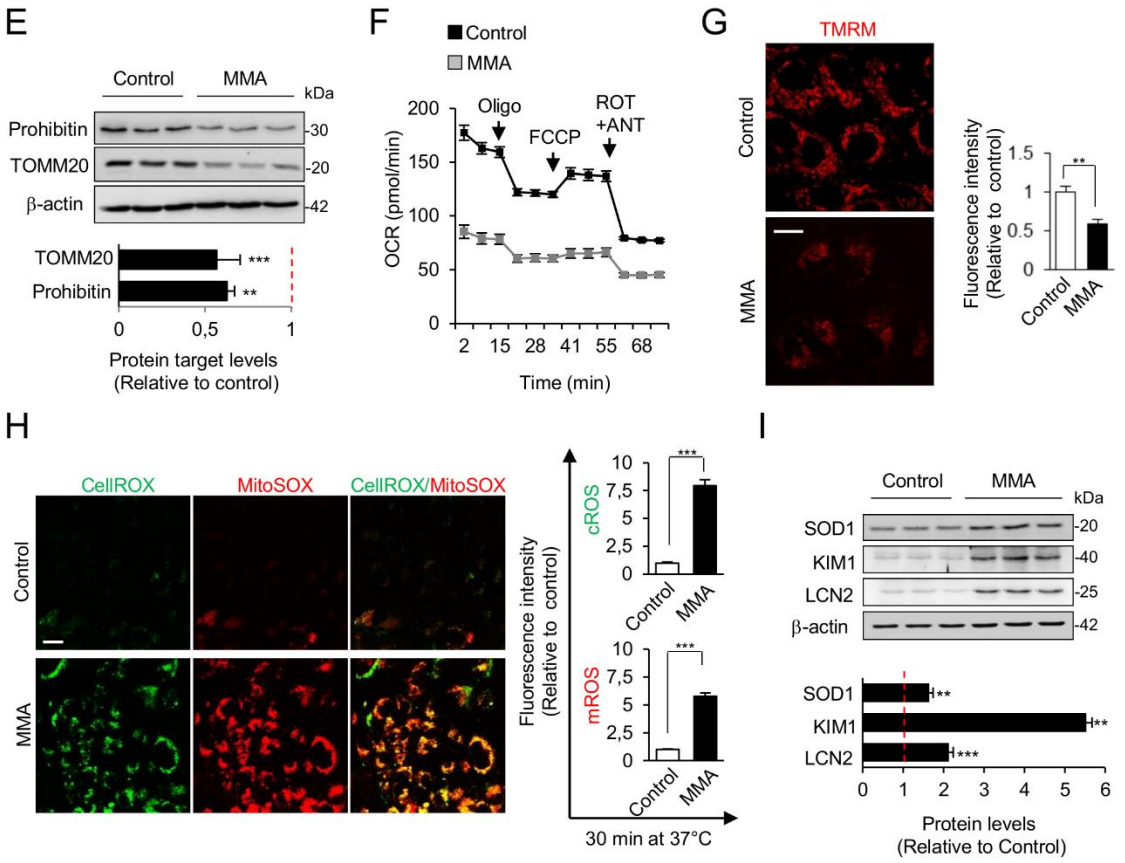

Figure 1 (ctd.) (**E**) Western blotting and densitometric analyses of prohibitin, TOMM-20 and prohibitin protein levels in control (n=3) and MMA cells (n=3). β-actin was used as a loading control. **P<0.01 and ***P<0.001 relative to control cells (**F**) Mitochondrial function of controls (n=3) and MMA patient-derived renal tubular cells (n=3). Oxygen consumption rates (OCRs) were measured at basal level and after the sequential addition of oligomycin (Oligo, 1µM), FCCP (0.5µM) and Rotenone (ROT; 1.0µM) + antimycin A (ANT; 1.0µM). The MMA patient-derived cells show a reduction in baseline respiration, ATP turnover and total respiratory capacity. (**G**) The cells were loaded with tetramethylrhodamine methyl ester (TMRM; 50nM for 30 min at 37°C) and analysed by live confocal microscopy to measure the resting mitochondrial membrane potential ($\Delta\psi_m$), which is significantly lower in MMA cells compared to control cells. Quantifications of TMRM fluorescence intensity obtained from 3 randomly selected fields per condition, with each containing ~10-15 cells. **P<0.01 relative to control cells. (**H**) Cells were loaded with CellROX (cellular ROS probe; 5 µM for 10 min at 37°C) and MitoSOX (mitochondrial ROS probe; 2.5 µM for 10 min at 37°C) and analysed by live confocal microscopy. Quantification of CellROX or MitoSOX fluorescence intensity was obtained from 3 randomly selected fields per condition, with each containing ~10-15 cells. ***P<0.001 relative to control cells. (**I**) Western blotting and densitometric analyses of SOD1, KIM1 and LCN2 protein levels in control (n=3) and MMA cells (n=3). ß-actin was used as a loading control. **P<0.01 and ***P<0.001 relative to control cells. The nuclei are counterstained with DAPI (blue). Yellow indicates co-localization in **C** and **H**. Scale bars: 10µm in **C**, 1µm in **D** and 10µm in **G** and **H**.

Autophagy is upregulated in renal tubular cells from MMA patients

Because the clearance of damaged/dysfunctional mitochondria occurs via autophagy, we speculated that dysregulated autophagy may explain the mitochondrial abnormalities in tubular cells from MMA patients. To investigate the state of autophagy, the cells were transduced with adenoviral particles expressing the green-fluorescent protein (GFP)-tagged autophagosome marker MAP1LC3 (GFP-LC3). We observed an increase in the number of autophagic vesicles in MMA cells when compared to controls (Fig. 2A). This observation was confirmed by quantifying the conversion of the LC3 (LC3I) to the autophagosome-associated lipidated form (LC3II; Fig. 2B) and by TEM (Fig. 2C).

Higher numbers of autophagosomes may reflect increased biogenesis or reduced lysosomal clearance. We next used the autophagy flux inhibitor Bafilomycin A1 (BfnA1), which prevents lysosomal degradation and reveals changes in autophagosome synthesis (Klionsky, 2016). The inhibition of lysosome function by BfnA1 treatment further increased both the numbers of punctate GFP-LC3 and the levels of LC3II in MMA cells (Figs. 2A-B), implying an increase in autophagosome biogenesis. The abnormal autophagic flux was not due to a block in autophagosomal delivery or lysosomal clearance because neither changes in autophagosomal-lysosomal fusion (as indicated by the colocalization of LC3-marked autophagosomes in LAMP1-flagged lysosomes and by Rab7 GTPase protein regulating the fusion process) nor in lysosomal dynamics (as scored by abundance of lysosomal proteins LAMP2 and cathepsin-D) nor in lysosomal degradation capacity (as indicated by Bodipy-FL-PepstatinA (PepA), a fluorescence-tagged PepA which binds to the active site of cathepsin-D in acidic lysosomes) occurred in MMA cells (Suppl. Figs. 5A-C).

When compared to control cells, MMA cells displayed increased levels of p62 (a protein regulating autophagic clearance of dysfunctional organelles), which was unrelated to increased mRNA levels (Fig. 2D; Suppl. Fig. 6 A-B). Consistently, ubiquitin (Ub)-positive inclusions accumulated in MMA cells (Fig. 2 E), substantiating a deregulation of autophagy. Similar to the changes in MMA patient derived renal tubular cells, there was a remarkable accumulation of LC3-marking autophagosomes and p62-forming aggregates in kidney biopsies from mut^0 patient (Fig. 2 F-G). These findings show that the abnormalities in the mitochondrial network are associated with dysregulation of autophagy in MMA cells *in vitro* and *in vivo*.

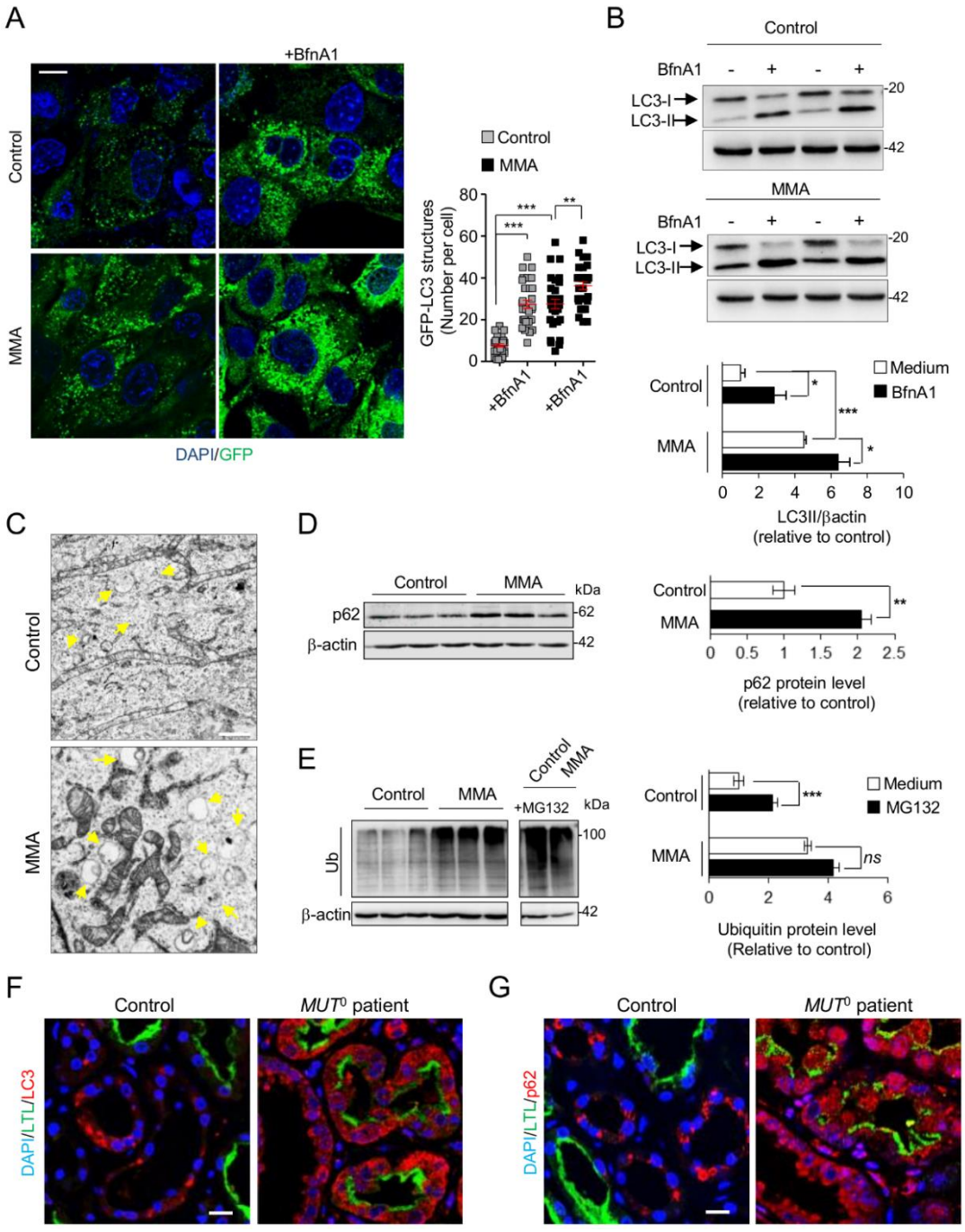

Figure 2. Dysregulation of autophagy in tubular cells derived from MMA patients.

(**A**) The cells were transduced with GFP-LC3B bearing adenoviral particles for 2 days and cultured in presence or in absence of 250 nM BfnA1 for 4h. Representative confocal micrographs (left panel)) and quantification of numbers of GFPLC3 structures in MMA and control cells (right panel; n=30 cells pooled from three control and MMA cells; each point representing the average number of GFPLC3^{+} vesicles in a cell). ***P<0.001 relative to control cells; **P<0.001 relative to untreated MMA cells. (**B**) Western blotting and densitometric analyses for LC3 protein levels in cells cultured in fed medium in presence or in absence of 250 nM BfnA1for 4h. *P<0.05 relative to controls or to MMA cells and ***P<0.01 relative to controls, n=3 independent experiments. (**C**) Representative images of transmission electron microscopy (TEM) showing increased numbers of autophagic vacuoles in MMA cells in comparison to controls. Yellow arrow head indicates autophagic vacuoles. (**D**) Western blotting and densitometric analyses of p62 protein levels in control and MMA cells. β-actin was used as a loading control. **P<0.01 relative to control cells (n=3). (**E**) Control and MMA cells were cultured in presence and in absence of 26S proteasome subunit inhibitor MG132 (50µM for 12h). Western blotting and densitometric analyses of ubiquitin protein levels. β-actin was used as a loading control. ***P<0.01 relative to control cells (n=3); ns, not significant. (**F-G**) Representative confocal micrographs of LC3 (**F**, red) or p62 (**G**; red) in LTL (green)-positive proximal tubules of human kidneys from control and MMA patient. Nuclei counterstained with DAPI (blue). Scale bars: 10µm in **A**, **F** and **G**, and 2µm in **C**.

Impaired mitophagy in renal tubular cells from MMA patients

The maintenance of the mitochondrial network is ensured through selective targeting of damaged mitochondria to autophagosomes via mitophagy (Pickrell, 2015). Given the higher LC3-fluxes in MMA cells experiencing mitochondrial dysfunction, we tested whether specific defects in mitophagy could account for the MMA-related alterations in mitochondrial network. The use of the mitochondrial stressor Rotenone, which leads to a rapid loss of $\Delta\psi_m$ and a major increase of ROS in the cells (Suppl. Fig. S7 A-B), allowed us to investigate the mitophagy cascade in both control and MMA cells. The rates of mitophagy were quantified by the subcellular distribution of TOMM20-labeled mitochondria within LC3-flagged autophagic structures. In control cells, only a small fraction of LC3-vesicles co-localized with mitochondria at baseline, whereas an increased number of mitochondria became coated with LC3 in response to rotenone ($\Delta\psi_m$ depolarization) (Fig. 3A, top panel). Conversely, the mitophagic response was severely blunted in MMA cells even after treatment with rotenone, indicating impaired relocation of damaged mitochondria to the autophagosome (Fig. 3A, bottom panel). The initiation of mitophagy requires a molecular signal from damaged mitochondria. Loss of mitochondrial $\Delta\psi_m$ inhibits the degradation of PINK1 and reroutes it to the surface of mitochondria, where it recruits parkin, tagging damaged mitochondria for autophagic degradation via ubiquitination (Youle, 2012). The baseline amount of PINK1and Parkin proteins were significantly decreased in MMA cells compared to controls (Fig. 3B), despite unchanged levels of mRNA transcripts (Suppl. Fig. S8).

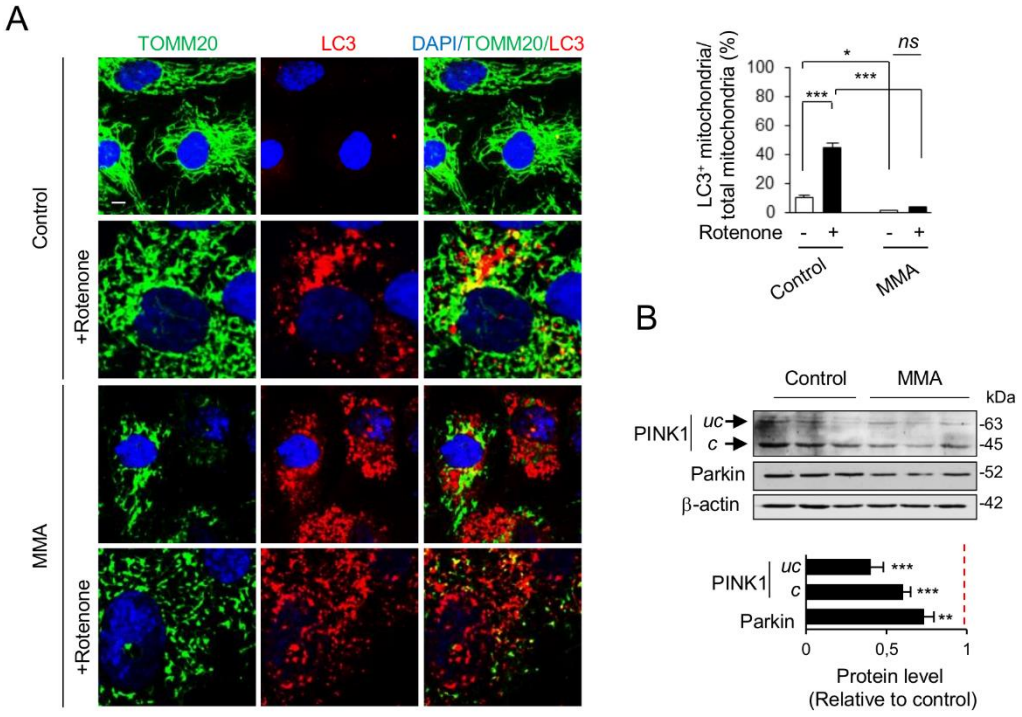

Figure 3. Defective PINK1/Parkin dependent clearance of damaged mitochondria in tubular cells derived from MMA patients.

(**A, C-D**) The cells were cultured in presence or in absence of Rotenone (10µM for 16 h). (**A**) Representative confocal micrographs and quantification of LC3/TOMM20 positive structures (in percentage of total mitochondria). Quantification obtained on 5 randomly selected fields per condition, with each containing ~10-15 cells; ***P<0.001 and *P<0.05 relative to untreated control cells; ***P<0.001 and relative to Rotenone-treated control cells; *ns*, not significant. (**B**) Western blotting and densitometric analyses of PINK1and parkin protein levels in control and MMA cells. β-actin was used as a loading control. **P<0.01 and ***P<0.01 relative to control cells (n=3). *uc*, uncleaved; *c*, cleaved. Scale bar: 10µm in **A**.

When exposed to rotenone, the control cells showed a significant increase in the recruitment of PINK1 to damaged mitochondria (colocalization with TOMM20), which was totally absent in MMA patient derived renal tubular cells (Fig. 3C).

Because PINK1 is retained on the outer mitochondrial membrane of depolarized mitochondria and disease relevant inactivating mutations in *MUT* led to depolarization of the mitochondrial membrane, we next investigated the stability of the full-length PINK1 (Wang, 2011). In control cells, rotenone-induced mitochondrial damage led to a major, 20-fold increase in the ratio of full-length to cleaved PINK1, which contrasted with the persistence of high level of cleaved PINK1 in the MMA cells (Fig. 3D). These findings collectively suggest an impairment of PINK1/Parkin mediated degradation of damaged/dysfunctional mitochondria in tubular cells derived from MMA patients.

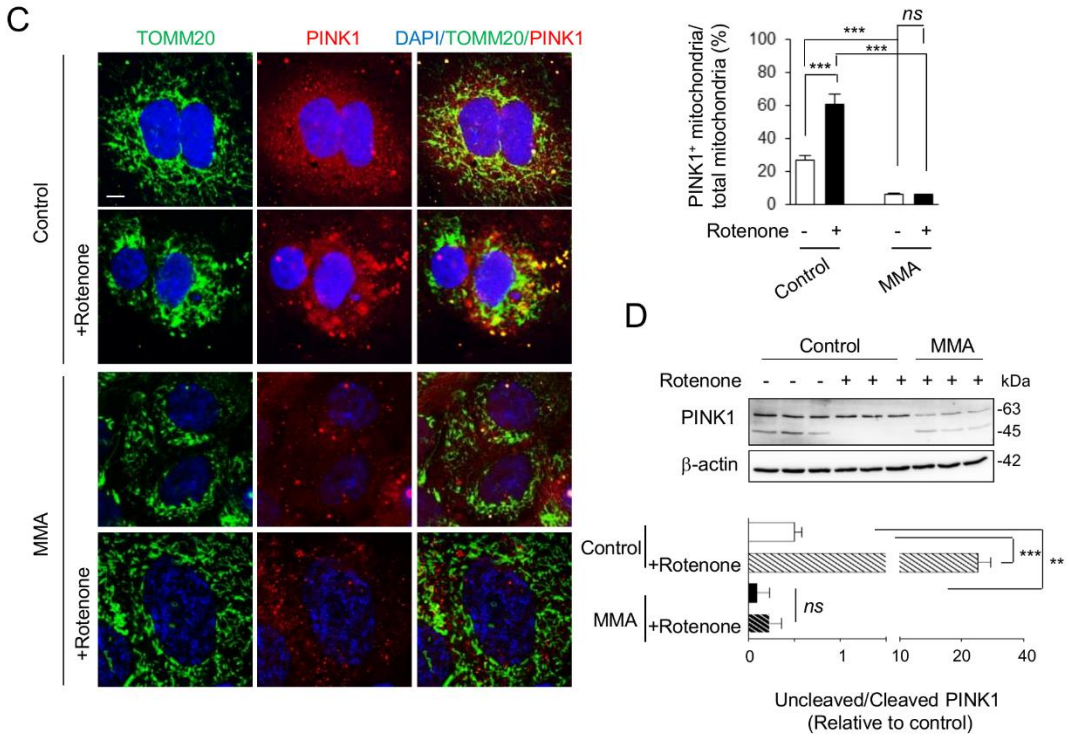

Figure 3 (ctd.) (**C**) Representative confocal micrographs and quantification of PINK1/TOMM20 positive structures (in percentage of total mitochondria). Quantification obtained on 5 randomly selected fields per condition, with each containing ~10-15 cells; ***P<0.001 relative to untreated control cells; ***P<0.001 relative to untreated-control cells; ***P<0.001 relative to Rotenone-treated control cells. *ns*, not significant. (**D**) Western blotting and densitometric analyses of PINK1protein levels in control and MMA cells. β-actin was used as a loading control. **P<0.01 and ***P<0.001 relative to control cells (n=3). *uc*, uncleaved; *c*, cleaved. Nuclei counterstained with DAPI (blue). Scale bar: 10µm in **C**.

Impaired mitochondrial homeostasis due to MUT deficiency causes epithelial damage

We next evaluated the direct link between the loss of MUT function, impaired mitochondrial homeostasis and dysfunction of renal tubular cells. We used primary proximal tubule cells (PTCs) derived from *Mut* $^{flox/flox}$ mice and an adenovirus-coding Cre-recombinase to investigate whether *Mut* deficiency may alter PINK1/Parkin-mediated mitophagy. The Cre-mediated loss of functional MUT, which was verified at both mRNA (Fig. 4A) and protein levels (Fig. 4B), lowered the mitochondrial mass (TOMM20 protein levels; Fig. 4B) and was reflected by an aberrant mitochondrial network with a prominent fragmented/rod-like shape evidenced by immunostaining for TOMM20 (Fig. 4C). These changes in mitochondrial mass and morphology were associated with a decrease in $\Delta\psi_m$ (as evidenced by TMRM staining and quantitative confocal imaging; Fig. 4D) and increase in mitochondrial ROS generation (Fig. 4E). Paralleling mitochondrial dysfunction, *Mut*-deleted cells showed a striking accumulation of autophagic cargoes and substrates (quantitation of LC3-marking autophagosomes and aggregates-forming p62) (Figs. 4 F-G) and decreased levels of PINK1 and Parkin (Fig. 4G).

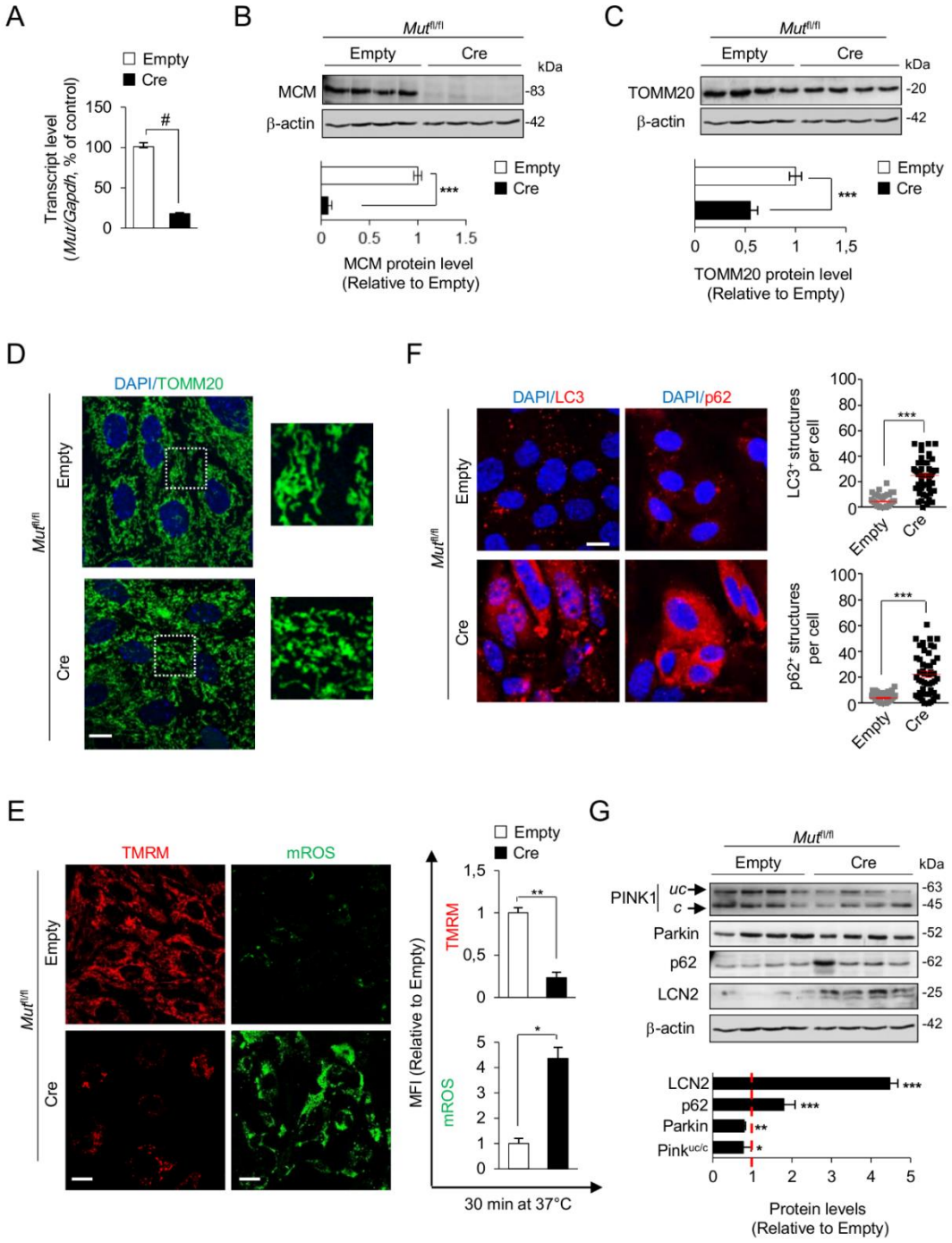

Figure 4. MUT deficiency impairs mitochondrial network and mitophagy, promoting cell damage in renal tubular cells.

Primary cultures of mouse proximal tubule cells were transduced with either empty (Empty) or Cre-recombinase (Cre) bearing adenoviral particles for 5 days. (**A**) The mRNA levels of *Mut* were analysed by real-time PCR., (n=4). (**B**-**C**) Western blotting and densitometric analyses of MUT (**B**) and TOMM20 (**C**) protein levels. β-actin was used as a loading control, (n=4). (**D**) The cells were immunostained with anti-TOMM20 (green) and analysed by confocal microscopy. (**E**) The cells were loaded with tetramethylrhodamine methyl ester (TMRM; 50nM for 30 min at 37°C) or with MitoSOX (mitochondrial ROS probe; 2.5µM for 10 min at 37°C), and analysed by live confocal microscopy. Quantifications of TMRM or MitoSOX fluorescence intensities obtained from 4 randomly selected fields pooled from four independent experiments, with each containing ~10-15 cells. (**F**) Representative confocal micrographs and quantification of LC3 (left panel) and p62 (right panel) positive structures in MUT-deficient PT cells (n=50 cells pooled from four independent experiments; each point representing the average number of $LC3^+$ or $p62^+$ vesicles in a cell). (**G**) Western blotting and densitometric analyses of PINK1, Parkin, p62 and LCN2 protein levels. β-actin was used as a loading control. uc, uncleaved; c, cleaved. **P*<0.05, ***P*<0.01, ****P*<0.001 and $^{\#}P$<0.0001 relative to cells transduced with empty adenoviral particles. Nuclei counterstained with DAPI (blue). Scale bars: 10µm in **D**, **E** and **F**.

Additionally, the alterations in mitochondrial network homeostasis and mitophagy were mirrored by the increase in the renal tubular marker lipocalin-2 (LCN2; Fig. 4G), suggesting that the maintenance of mitochondrial network integrity is essential for preserving homeostasis in renal tubule cells.

Impaired mitochondrial homeostasis leads to kidney damage in Mut$^{ko/ki}$ mice

To enhance the translational potential of the findings, we investigated the changes in mitochondrial network homeostasis and mitophagy in the kidneys of compound heterozygous *Mut*$^{ko/ki}$ mice, a novel model of MMAuria (Forny, 2016). The protein expression of MUT was 5-fold reduced in the kidneys of the *Mut*$^{ki/ko}$ mice (Fig. 5A), consistent with a major reduction in the MUT enzymatic activity in these kidneys (Forny, 2016). Immunofluorescence, TEM and western blotting analyses indicated that the mitochondrial network, which appeared fragmented and with disorganized cristae, was reduced in the epithelial cells lining the renal tubules of the *Mut*$^{ki/ko}$ mice in comparison to control littermates (Figs. 5 A-C).

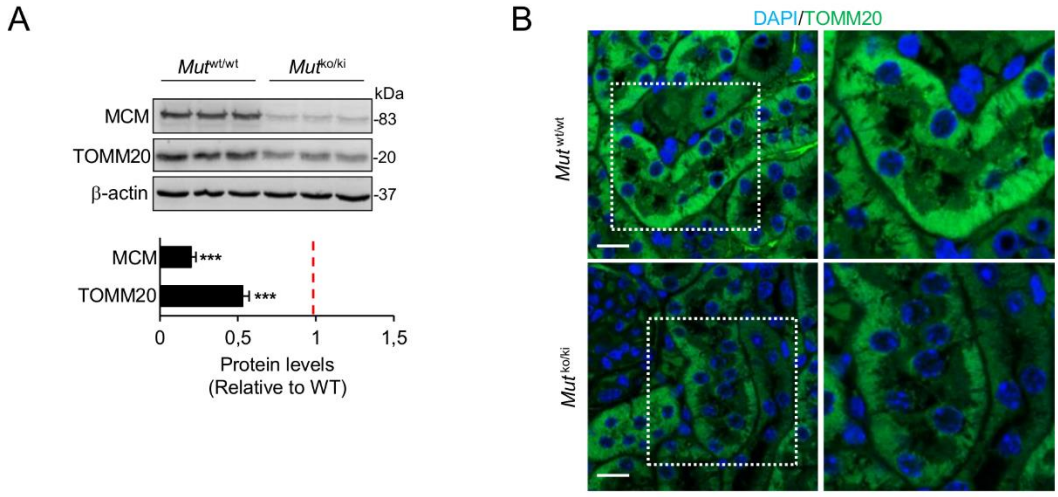

Figure 5. Altered mitochondrial homeostasis and impaired mitophagy leads to kidney damage in *Mut*$^{ko/ki}$ mice.

(**A**) Western blotting and densitometric analyses of MUT and TOMM20 protein levels in whole kidney lysates from *Mut* mice. (**B**) Representative confocal micrographs of TOMM20 in *Mut* mouse kidneys.

LC3-flagging autophagosomes or p62-positive aggregates accumulated in the kidneys of the *Mut*$^{ki/ko}$ mice (Figs. 5 D-E) pointing at induction of autophagy. Similar to mitophagy defects observed in MMA patient-derived and *Mut*-deleted renal tubular cells, the mitophagy proteins PINK1and Parkin were strikingly lower in the kidneys from *Mut*$^{ko/ki}$ mice when compared to control littermates (Fig. 5F).

These changes were paralleled by an increase in LCN2 (Fig. 5F) as well as by abnormal plasma and urine parameters (Table 2) indicating MMA-associated renal tubular damage.

Consistent with *in vitro* findings (MMA cells and genetic deletion of *Mut*), these *in vivo* data suggest that MUT deficiency leads to renal tubule dysfunction by compromising PINK1/Parkin mediated mitophagy and mitochondrial homeostasis.

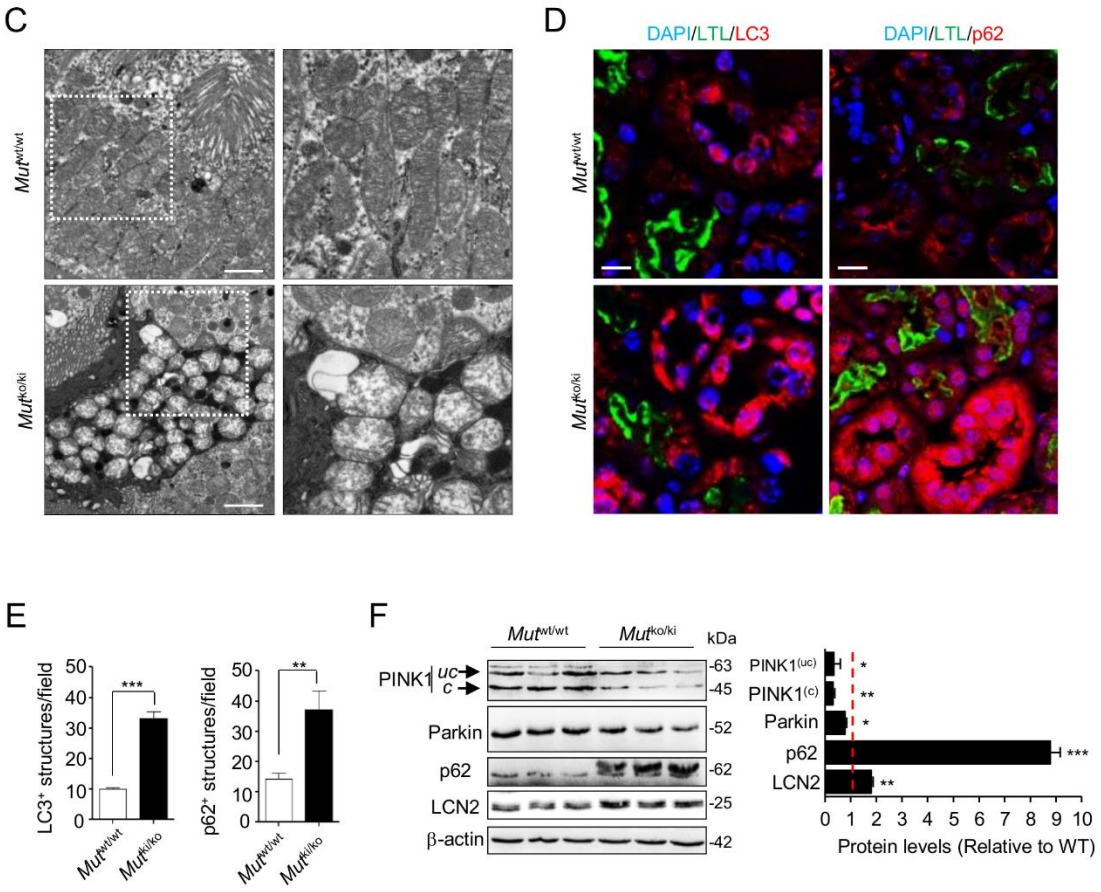

Figure 5 (ctd.) (**C**) TEM micrographs showing filamentous mitochondria with preserved cristae organization in the kidneys of *Mut*$^{wt/wt}$ mice vs. fragmented mitochondria with destroyed cristae organization in the kidneys of *Mut*$^{ki/ko}$ mice. (**D-E**) Representative confocal micrographs and quantification of the numbers of LC3 (left panel; red) or SQSTM1structures (right panel; red) per field in stained sections; n= 5 fields per kidney slices. (**F**) Western blotting and densitometric analyses of PINK1, Parkin, p62 and LCN2 protein levels in whole kidney lysates from *Mut* mice. *uc*, uncleaved; *c*, cleaved. **P*<0.05, ***P*<0.01 and ****P*<0.001 relative to control littermates. Nuclei counterstained with DAPI (blue). Scale bars: 10µm in **B**, **D,** and 1 µm in **C**.

Table 2. Clinical and biochemical parameters in *Mut*$^{wt/wt}$ and *Mut*$^{ko/ki}$ mice.

	***Mut*$^{wt/wt}$**	***Mut*$^{ki/ko}$**	*P* value
Body weight (g)	23.1 ± 0.34	21.4 ± 0.36	≤ 0.01
Food intake (per g BW)	0.16 ± 0.05	0.11 ± 0.01	≤ 0.05
Water intake (per g BW)	0.26 ± 0.05	0.11 ± 0.01	≤ 0.01
Plasma			
Creatinine (mg/dl)	0.11 ± 0.03	0.11 ± 0.03	*ns*
BUN (mg/dl)	43.51 ± 4.98	61.99 ± 1.59	≤ 0.001
Urine			
Diuresis (µl/h/g BW)	10.47 ± 1.31	1.46 ± 0.24	≤ 0.001
Sodium (mM mM^{-1} creatinine)	32.85 ± 1.20	40.58 ± 2.22	≤ 0.001
Chloride (mM mM^{-1} creatinine)	51.97 ± 2.96	60.40 ± 3.81	≤ 0.07
Potassium (mM mM^{-1} creatinine)	71.08 ± 2.76	85.54 ± 2.88	≤ 0.01
Calcium (mg mg^{-1} creatinine)	0.02 ± 0.003	0.19 ± 0.03	≤ 0.001
Phosphate (mg mg^{-1} creatinine)	3.66 ± 0.30	6.29 ± 0.41	≤ 0.01
Magnesium (mg mg^{-1} creatinine)	1.59 ± 0.01	2.56 ± 0.16	≤ 0.01
Metabolic papameters			
C3 (mmol/l)	0.03 ± 0.002	0.25 ± 0.02	≤ 0,001
MMA excretion (mmol/mol creat)	10 ± 2.5	10000 ± 250	≤ 0.001

Table 2: *Mut*$^{wt/wt}$ (n=5) and *Mut*$^{ki/ko}$ (n=5) female mice on C57BL/6 background at 16 weeks of age on normal diet. Values are given as mean ± SEM. *ns*= non-significant.

Targeted antioxidant treatment restores mitochondrial homeostasis and attenuates cellular damage in MMA cells

Finally, we explored the functional consequences of neutralizing the excess of mitochondrial oxidative stress by treating MMA patient-derived cells with the mitochondria-targeted anti-oxidant Mito-TEMPO (MT, 10µM for 24h) (Lood, 2016). This treatment rescued the mitochondrial morphology and mass (Figs. 6 A-B), restored the mitochondrial membrane potential ($\Delta\psi_m$, Fig. 6C) and neutralized the excessive production of ROS (Fig. 6C).

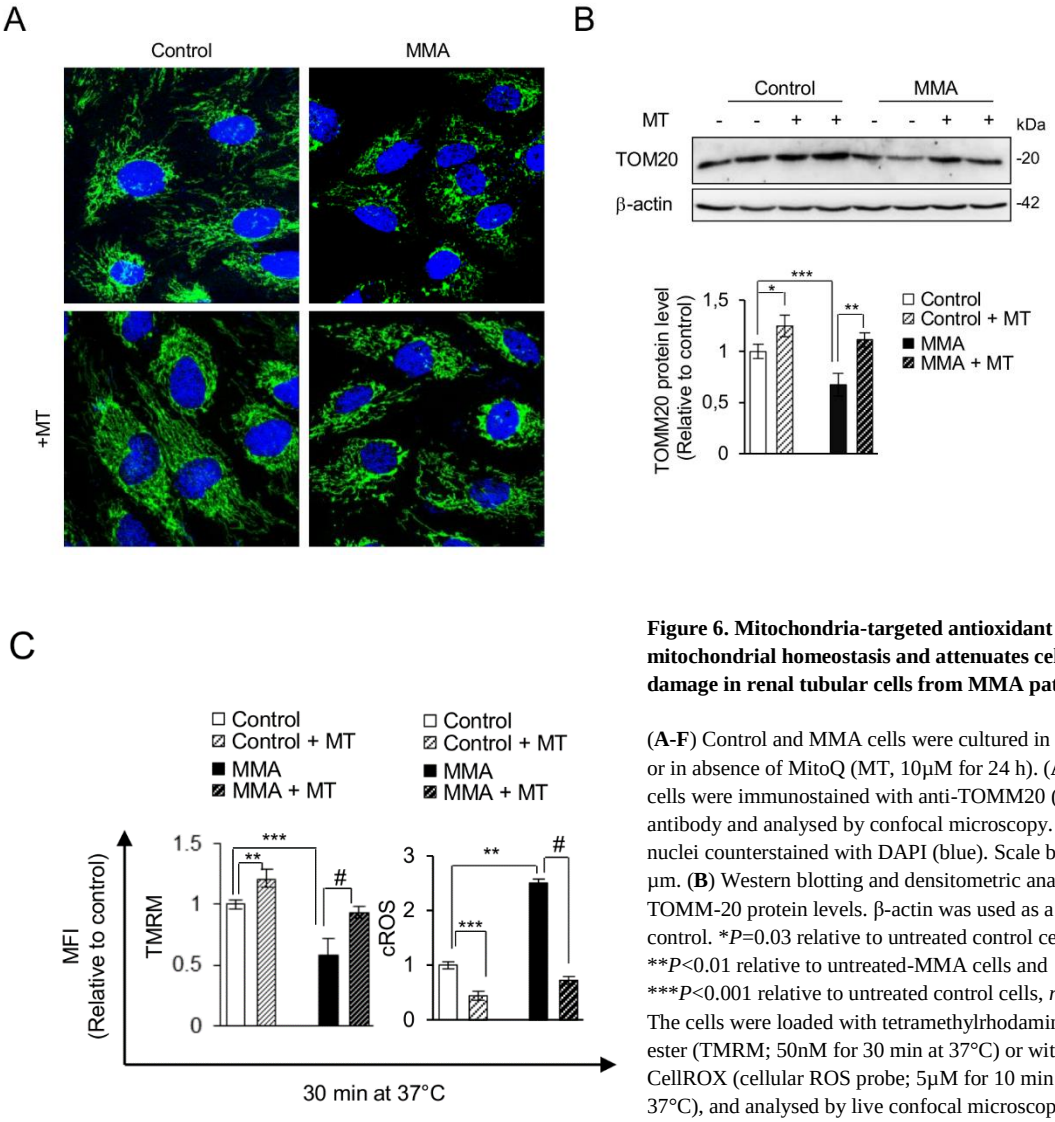

Figure 6. Mitochondria-targeted antioxidant restores mitochondrial homeostasis and attenuates cell damage in renal tubular cells from MMA patients.

(**A-F**) Control and MMA cells were cultured in presence or in absence of MitoQ (MT, 10µM for 24 h). (**A**) The cells were immunostained with anti-TOMM20 (green) antibody and analysed by confocal microscopy. The nuclei counterstained with DAPI (blue). Scale bar, 10 µm. (**B**) Western blotting and densitometric analyses of TOMM-20 protein levels. β-actin was used as a loading control. **P*=0.03 relative to untreated control cells, ***P*<0.01 relative to untreated-MMA cells and ****P*<0.001 relative to untreated control cells, *n*=3. (**C**) The cells were loaded with tetramethylrhodamine methyl ester (TMRM; 50nM for 30 min at 37°C) or with CellROX (cellular ROS probe; 5µM for 10 min at 37°C), and analysed by live confocal microscopy. Quantifications of TMRM or MitoSOX fluorescence intensities obtained from 4 randomly selected fields pooled from four independent experiments, with each containing ~10-15 cells. ***P*<0.01 and ****P*<0.001 relative to untreated control cells, and $^{\#}$*P*<0.001 relative to untreated MMA cells; *n*=3.

Importantly, treatment with MT decreased either the p62 protein amounts or ubiquitin-positive inclusions (Fig. 6C), and partially increased the levels of PINK1 (Fig. 6D), suggesting that MMA cells can effectively clear damaged/dysfunctional mitochondria targeted for mitophagy. Notably, the replenishment of mitochondrial network homeostasis by Mito-TEMPO (MT) was mirrored by a marked decrease in the protein levels of LCN2 (Fig. 6E), indicating that the excess of mitochondrial ROS production resulting from impaired targeting of damaged mitochondria to mitophagy is a decisive factor in epithelial damage associated with in MMA.

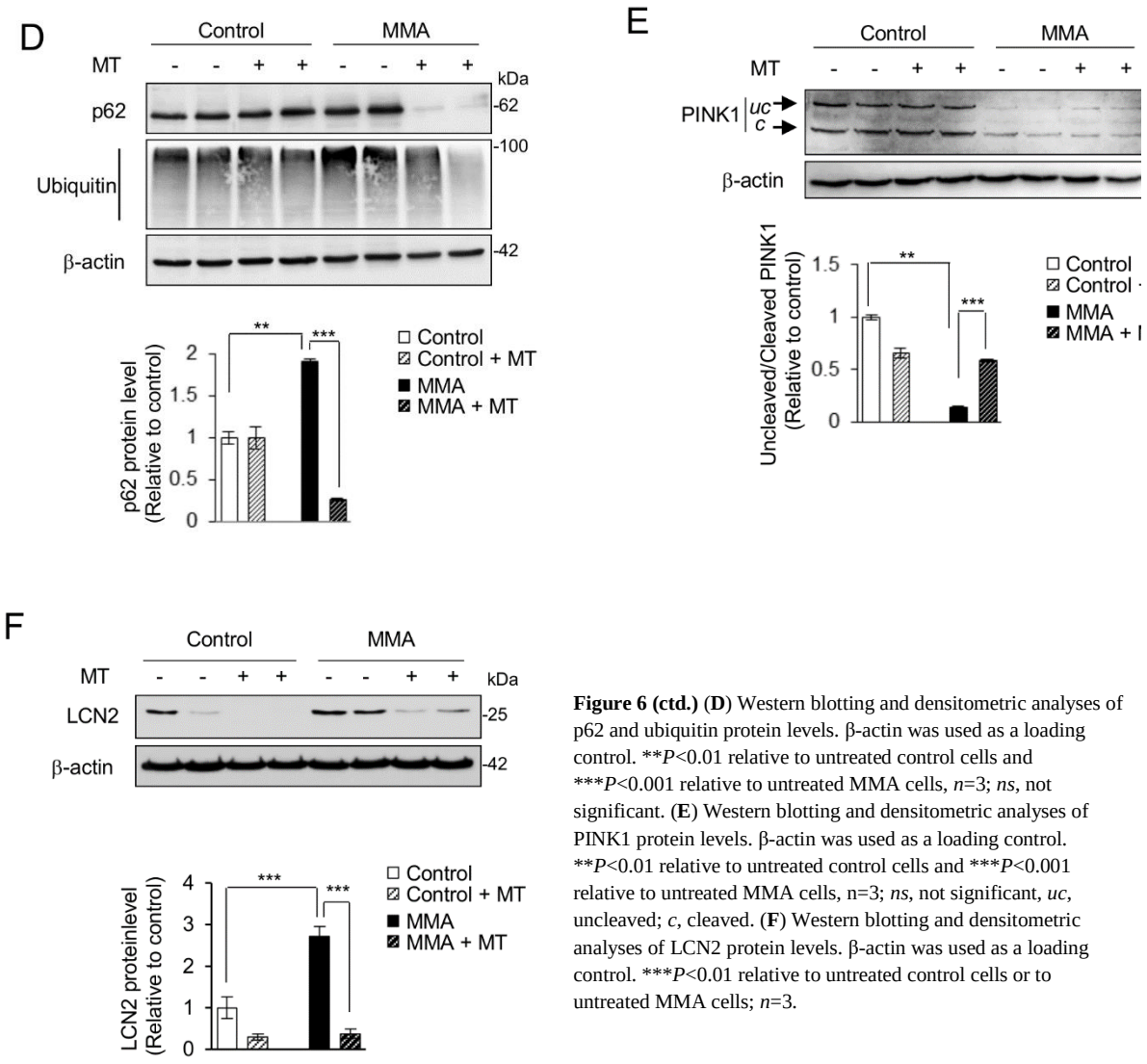

Figure 6 (ctd.) (**D**) Western blotting and densitometric analyses of p62 and ubiquitin protein levels. β-actin was used as a loading control. ***P*<0.01 relative to untreated control cells and ****P*<0.001 relative to untreated MMA cells, *n*=3; *ns*, not significant. (**E**) Western blotting and densitometric analyses of PINK1 protein levels. β-actin was used as a loading control. ***P*<0.01 relative to untreated control cells and ****P*<0.001 relative to untreated MMA cells, n=3; *ns*, not significant, *uc*, uncleaved; *c*, cleaved. (**F**) Western blotting and densitometric analyses of LCN2 protein levels. β-actin was used as a loading control. ****P*<0.01 relative to untreated control cells or to untreated MMA cells; *n*=3.

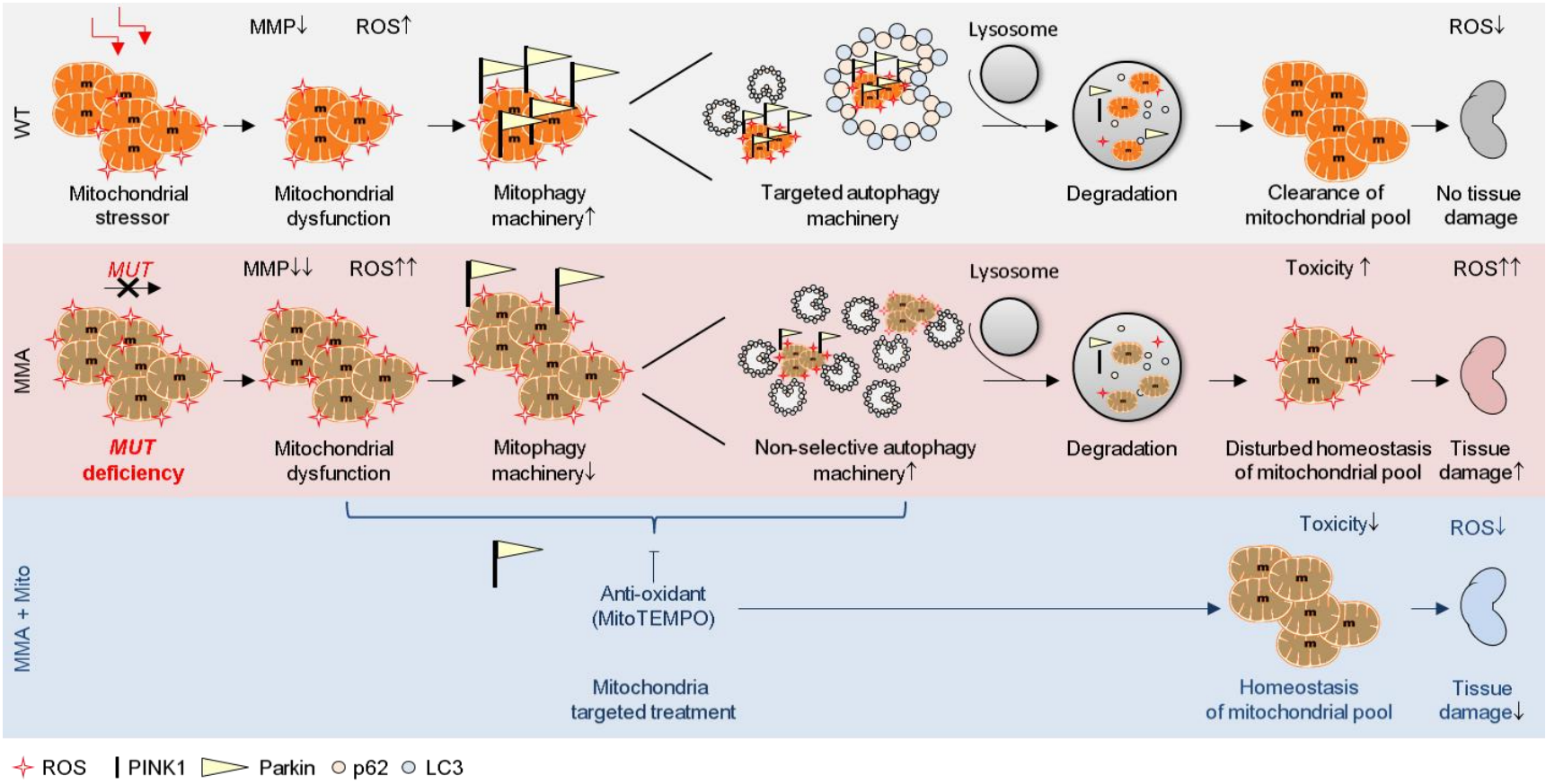

Figure 7. Synopsis

Schematic representation of the main findings: Deficiency of the *Mut* gene leads to altered mitochondrial integrity and function in different human and mouse *in vitro* and *in vivo* models. MQC is an essential mechanism to sustain cellular homeostasis in stressful conditions and autophagy plays a leading role in the removal of damaged mitochondria. Our data suggests, that autophagy is highly active in our different MMA models. Selective autophagic removal of damaged mitochondria (=mitophagy) is dysregulated in the *Mut*-deficient systems leading to activation of autophagy and defective mitochondrial homeostasis and kidney damage in MMA. Mitochondrial targeted anti-oxidant treatment by MitoTEMPO reverses the obtained findings. The findings highlight the mitochondrion as key organelle in MMA and point at excessive mROS production as a major disease driving burden. (MMP=mitochondrial membrane potential, mROS=mitochondrial reactive oxygen species).

3. Discussion

Mitochondria form a dynamic, interconnected network that integrate cell metabolism by regulating anabolic and catabolic functions (Nunnari, 2012). Congenital defects in mitochondria-localized proteins, as exemplified by MMA, cause epithelial cell damage resulting in end-organ dysfunction. By combining genetic and pharmacological approaches, we demonstrate that mitochondria are fragmented and dysfunctional in human and mouse renal cells lacking functional MUT. Through the dysregulation of the PINK1/Parkin pathway, disease-causing *MUT* mutations or *Mut* gene deficiency impair the autophagy-mediated clearance of dysfunctional mitochondria. In turn, the cells accumulate autophagy/ubiquitinated proteins and mitochondrial ROS, causing cell damage. These data reveal the mechanism bridging mitochondrial dysfunction and renal epithelial cell damage in MMA and the importance of PINK1/Parkin dependent mitochondrial turnover in epithelial cell homeostasis. They also indicate that targeting mitochondrial ROS production offers therapeutic perspectives to reduce epithelial damage downstream to primary mitochondrial defects.

Our findings show that MUT deficiency leads to a major alteration in mitochondrial integrity and function, as evidenced by decreased numbers of swollen mitochondria, which appear fragmented and with disorganized cristae; altered mitochondrial membrane potential-a key requisite for effective oxidative phosphorylation and energy production; and impaired bioenergetics profiling, as evidenced by a reduction in baseline respiration, ATP turnover and total respiratory capacity. These changes were mirrored by excessive mitochondrial ROS production, resulting in oxidative stress and renal epithelial damage in both *Mut*-depleted renal tubular cells and MMA patient-derived cells. Similar changes in the mitochondrial network were observed in the kidneys of the $Mut^{ko/ki}$ mice, a novel mouse model recapitulating the kidney damage associated with MMA (Forny, 2016), extending earlier observations in transgenic Mut mice rescued from neonatal lethality (Manoli, 2013) and in a single kidney biopsy of a mut^0 patient (Zsengellér, 2014). Taken together, these findings point to mitochondria as key actors in driving cellular damage in MMA. The question was how MUT deficiency impacts mitochondrial homeostasis and how mitochondrial dysfunction contributes to epithelial damage.

Mitochondrial homeostasis is crucial for generating the high, local energy demands which are requested for the maintenance of cellular function, particularly in tissues with high reabsorptive activities like the renal tubules (Emma, 2016). As a result, a variety of inter-connected mechanisms, known as MQC (MQC), have evolved to regulate mitochondrial morphology, content and function (Kotiadis, 2014; Fisher, 2012). The removal of damaged mitochondria by autophagy is an essential component of MQC (Ni, 2015; Fisher, 2012). In fact, autophagy-mediated turnover of damaged mitochondria is required for protecting renal tubules from injury (Isaka, 2011), whereas deletion of essential autophagy genes damages renal tubular cells through defective mitochondrial clearance and increased reactive oxygen species (ROS) (Yamamoto, 2016).

In order to investigate MQC, we first assessed the state of autophagy in cells lacking MUT. Our studies reveal a robust, consistent increase in the number of autophagic vesicles and in the levels of autophagosome-associated LC3-II and autophagy substrate p62/SQSTM1 in the different models of MUT deficiency, including tubular cells from MMA patients, mPT cells from *Mut*$^{flox/flox}$ mouse kidneys, kidneys of *Mut*$^{ko/ki}$ mice and kidney biopsies from a patient with MMA. We furthermore observed an excessive autophagic vacuole formation by TEM, sustaining the idea of an upregulated state of autophagy. Indeed, upregulated autophagy has been shown to be protective in models of acquired renal tubular injury (Liu, 2014; Isaka, 2011).

Dysregulation of autophagy may occur at any steps involved in autophagosome formation and/or can be induced by factors that regulate autolysosomal trafficking or fusion between different subcellular compartments. For instance, accumulation of LC3-positive organelles unable to fuse with lysosomes has been observed in different lysosomal storage diseases (LSD), (Ballabio, 2009). However, when treating MMA cells with BfnAI, which accurately measures autophagy flux (Bento, 2016), we observed a substantial co-localization of LC3-positive vesicles with the lysosome marker LAMP1, suggesting that the delivery and the autophagosome-lysosome fusion is not compromised in MMA cells. Therefore, we postulated that MUT deficiency is likely to affect autophagy either by increasing autophagosome biogenesis or by regulating the lysosomal degradation capacity. The latter hypothesis does not sound valid, because neither lysosomal dynamics (as scored by the abundance and positioning of the lysosomal proteins LAMP2 and CtsD) nor lysosomal activity (as measured by the

fluorescent tagged PepstatinA which binds active CtsD within mature lysosomes) appeared to be affected by MUT depletion. Instead, treatment with the lysosome inhibitor BfnAI increased the number of autophagosomes and the levels of the LC3-II in control and in MMA cells - clearly indicating that MUT deficiency may induce autophagosome synthesis rather than compromise their clearance.

Given the central role that autophagy plays in responding to cellular stresses, the higher LC3 fluxes in MUT-deficient cells experiencing mitochondrial dysfunction support the concept that these cells need autophagic functions or products to cope with stress. In this scenario, damaged and dysfunctional mitochondria as observed in MUT-deficient cells should be removed by autophagosomal sequestration and subsequently degraded by lysosomes, clearing the cells of excessive dysfunctional mitochondria and their toxic byproducts (Twig, 2008). Particularly, the selective autophagic removal of damaged mitochondria (also known as mitophagy) relies on molecular sensors that detect potentially dangerous cues, converting them into signals that are ultimately relayed to the autophagic machinery. A recently identified paradigm pathway for mammalian mitophagy is mediated by the kinase PINK1 and the E3 ubiquitin ligase Parkin, which collaborate to sense and trigger the removal of damaged mitochondria (Valente, 2004; Kitada, 1998). Studies in Drosophila further suggested a role for PINK1 and Parkin in the regulation of mitochondrial integrity, since the loss of either protein results in mitochondrial dysfunction, driving degeneration of flight muscles and dopaminergic neurons (Greene, 2003; Park, 2005).

In healthy mitochondria, PINK1 is constitutively imported to the inner mitochondrial membrane, where it is cleaved by several proteases and proteolytically degraded (Greene, 2012). In case of impaired mitochondrial membrane potential, the degradation of PINK1 is inhibited and the protein is rerouted to the surface of the mitochondria, where it accumulates and recruits Parkin (Kim, 2008; Nunnari, 2012). During mitophagy, PINK1 and Parkin cooperate to attach ubiquitin tags to proteins on the outer membrane of depolarized mitochondria, tagging them for autophagic removal and degradation. The use of Rotenone, a well-established toxin which depolarizes mitochondria and increases mitochondrial ROS levels, allowed us to track mitophagy events and autophagic sequestration of damaged mitochondria in renal tubular cells. Consistently, treatment with Rotenone

decreased the cleavage of PINK1 maintaining full-length PINK1 onto dysfunctional mitochondria (as marked by TOMM20) thus initiating the sequestration by autophagy (as shown by colocalization between damaged TOMM20 positive mitochondria and LC3-positive autophagosomes) in normal cells. However, this mitophagy signal was defective in MUT-deficient cells experiencing mitochondria dysfunction, with decreased protein levels of PINK1 and Parkin at baseline and a lack of proper recruitment of full-length PINK1 to the mitochondrial membrane upon Rotenone treatment. As a result, the damaged mitochondria are not flagged for disposal despite the abundant levels of p62, a stress-induced ubiquitin binding protein that targets damaged mitochondria to autophagosomes (Cohen-Kaplan, 2016). Taken together, these findings indicate that dysregulation of PINK/Parkin mediated mitophagy is explaining the abnormal accumulation of dysfunctional mitochondria in MMA and MUT-deficient systems.

The link between defective mitochondrial homeostasis characterizing MMA and organ damage is supported by elevated levels of kidney damage markers (LCN2, KIM1) and disturbed metabolic and renal function parameters in the $Mut^{ki/ko}$ mouse. A similar link between dysfunctional mitochondria, loss of PINK1 and tissue damage has recently been described in a human pulmonary fibrosis model (Bueno, 2014). Tubulointerstitial lesions frequently observed in kidney biopsies of MMA patients progressing to fibrosis underline the importance of that finding (Rutledge, 1993; Goyenechea, 2012). To substantiate the causal link between MUTdeficiency and the observed findings, we performed Cre mediated *Mut* deletion in renal tubular cells *in vitro*. The deletion of MUT was able to reproduce all observed findings including changes in mitochondrial homoeostasis, loss of PINK1 and Parkin expression, accumulation of autophagic marker proteins and elevated markers for kidney damage including LCN2. This strongly supports the importance of the MUT enzymatic activity for mitochondrial homoeostasis, regulation of autophagy and oxidative stress and provides causal evidence linking MMA to renal tubular damage. The carrier protein lipocalin-2 (LCN2) is of particular interest, since it has been shown as essential for chronic kidney disease progression in mice and humans (Viau, 2010), by mechanisms involving the mitogenic effects of the epidermal growth factor receptor (EGFR) and apoptosis through ROS generation (El Karoui, 2015). The fact that LCN2 is

upregulated by the inactivation of MUT *in vitro* and *in vivo* provides a strong candidate to mediate kidney damage in MMA.

The potential importance of mitochondrial ROS production for cellular damage in MMA led us to investigate the beneficial effect of mitochondria-targeted antioxidant therapy in human renal cells from MMA patients and controls. MitoTEMPO (MT) is an orally bioavailable compound that predominantly accumulates within the mitochondria, due to the covalent binding of a lipophilic cation (triphenylphosphonium, TPP) to the endogenous antioxidant CoQ10. It exerts a direct antioxidant action, by scavenging peroxyl, peroxynitrites and superoxide in the mitochondria. Interest for mitochondria-targeted antioxidants, and MT in particular, has been triggered by *in vivo* studies showing protection in a number of clinical situations associated with oxidative stress, including sepsis, ischemia-reperfusion injury, liver disease, neurodegenerative diseases, and hypertension (Jin, 2014). Treatment of MMA patient-derived cells with MT, which efficiently neutralized the ROS production, restored mitochondrial morphology, normalized mitochondrial mass, and also increased the ratio of uncleaved vs. cleaved PINK1 ratio, promoting autophagic degradation of dysfunctional mitochondria. Importantly, the beneficial effects of treatment with MT were reflected by a robust reduction of LCN2 levels, lending support to the idea that ROS play a major role in the development of MMA-associated kidney damage. Although these promising results should be confirmed in animal models, they provide new therapeutic perspectives for directly reducing the consequences of mitochondrial damage due to MUT deficiency.

In conclusion, we identified a novel cascade connecting primary mitochondrial disease, impaired PINK/Parkin mediated autophagic degradation of dysfunctional mitochondria, oxidative stress and tubular cell damage in MMA. By identifying this mechanism and showing that mitochondria-targeted antioxidant treatment restores mitochondrial homeostasis and decreases mediators of tissue damage, we provide a novel strategy for treating renal tubular dysfunction in MMA and mitochondrial disorders.

4. Material and Methods

Antibodies and reagents. The following antibodies were used in the study: mouse anti-Methylmalonyl Coenzyme A mutase antibody (67869 Abcam, Cambridge, UK), rabbit anti-Aquaporin 1 antibody (2219, Millipore, Billerica, USA), rabbit anti-Aquaporin 2 antibody (A7310, Sigma, Saint Louis, USA), rabbit- anti-Kidney injury molecule 1 (KIM1) antibody (76701, Novus Biologicals, Littleton, USA), rabbit anti-LC3 antibody (PM036, MBL, Woburn, USA), sheep anti-UMOD (K90071C, Meridian Life Science, Kampenhout, Belgium), rabbit anti-p62 antibody (PM045, PM036, MBL, Woburn, USA), rabbit anti-prohibitin (28172, Abcam, Cambridge, UK),rabbit anti-TOMM20 (11415, Santa Cruz Biotechnology, Dallas, USA)l, rabbit anti-Parkin (15954, Abcam, Cambridge, UK), goat anti-PINK1 (32584, Santa Cruz, CA, USA), rat anti-Lamp1 (sc-19992, Santa Cruz Biotechnology), goat anti-Cathepsin-D (sc-6486, Santa Cruz Biotechnology), rabbit anti-Rab7 (126712, Abcam, Cambridge, UK), mouse-anti-Ubiquitin (sc-8017, Santa Cruz Biotechnology), rabbit anti-Lipocalin-2(ab63929, Abcam, Cambridge, UK), rabbit-anti superoxide dismutase (SOD)-1 (13498, Abcam, Cambridge, UK) and mouse anti-β-actin antibody (A5441, Sigma, Saint Louis, USA). Compounds included Bafilomycin-A1 (ALX-380-030, Enzo Life Sciences, Zurich, Switzerland), Rotenone (R8875, Sigma, Saint Louis, USA), MG132 (474790, Merck Millipore, Zurich, Switzerland) and mito-TEMPO (MT, ALX-430-150-M005, Enzo Life Sciences, Zurich, Switzerland). The adenovirus particles carrying green fluorescent protein (GFP) microtubule-associated protein light chain 3(LC3B) and Cre-recombinase were purchased from Vector Biolabs (USA).

Human kidney biopsies. The human kidney biopsies were obtained from a 18 year old, female MMA patient harboring a mutation in the *MUT* gene and from one control (non-transplanted, normal control kidney). Informed consent was obtained and the use of the human biopsy samples was approved by the Ethical Review Board of Saint-Luc Academic Hospital (Brussels, Belgium) and the EURenOmics consortium (FP7, 2007–2013, grant agreement no. 305608).

Cell culture. The renal tubular cells were obtained from urines of healthy donors or patients with mut^0 (Ruppert, 2015). The cells were cultured in DMEM GlutaMAX (Gibco, Luzern, Switzerland) and supplemented with 10% fetal calf serum (Biochrome, Berlin, Germany) and Pen/Strep (100 U/ml

penicillin, Gibco, Luzern, Switzerland). For inducing mitochondrial damage, the cells were cultured in media containing 10µM Rotenone for 16h and then processed as described above. For autophagy flux analysis, 250nM BafilomycinA1 or corresponding vehicle (that is water) were then added to cell medium for 4h. For proteasome inhibition, the cells were cultured in media containing 50µM Rotenone for 6h and then processed as described above. For neutralizing the excessive generation of mitochondrial oxidative stress, the cells were cultured in media containing 10mM MT for 24h and processed as described above. For expression studies, the cells were transduced using adenovirus constructs carrying mouse GFP-MAP1LC3B (Ad-GFP/mMAP1LC3B) and analysed by confocal microscopy.

Enzymatic activity. The cells derived from human urine were classified as mut^0 mutations by assessing MUT activity as previously described (Baumgartner, 1983). Briefly, the crude cell homogenates were sonicated and 1 mM DL-2-(methyl-^{14}C)methylmalonyl-CoA (ARC; specific activity 7.03 MBq/mmol in assay) was added in absence (holo-MUT activity) and presence (total MUT activity) of the cofactor Adenosylcobalamin (AdoCbl) 50 µM)) in darkroom safelight red conditions. The reaction was terminated by the addition of 5N KOH (Merck, Darmstadt, Germany). The samples were enriched with succinic acid (Merck, Darmstadt, Germany) to visualize the succinate peak during HPLC separation. Succinate and methylmalonate peaks were detected at 210 nm by an UV detector. Quantification of the (^{14}C) succinate fraction was performed with Optiphase HiSafe2 counting cocktail (PerkinElmer) in a Tri-Carc C1 900TR scintillator spectrometer (Packard). The protein concentration of the cell lysates was determined using the Lowry method. The MUT activity is given as pmol succinate which is formed per minute and mg protein (pmol/min/mg). Both holo-MUT (assay without AdoCbl) and total MUT (assay with 50–100 M AdoCbl) activities were strongly decreased in cells obtained from MMA patients whereas total MUT activity was normal in the cells derived from healthy controls.

Cell viability assay. Cell viability was determined by the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Rockville, USA). Cells were seeded at a density of 5000 cells/well of 96 well plate and grown until they reached 90% confluence. The cell medium was replaced by water-soluble tetrazolium

salt solution and incubated for 30 min at 37°C.according to the manufacturer's protocol. The amount of formazan dye generated by dehydrogenase activity was measured at 450 nm in a TECAN infinite 200 reader (Männedorf, Switzerland) in according to the manufacturer's specifications.

Mice. The mice were maintained under temperature-and humidity-controlled conditions with 12-h light/12-h dark cycles with free access to appropriate standard diet in accordance with the institutional guidelines of National Institutes of Health Guide for the Care and Use of Laboratory Animals. The mice bearing *Mut*^{flox} allele, in which exon 3of the *Mut* gene is flanked by 2 *loxP* sequences, and the mice carrying M698K point mutation in the *Mut* gene was performed by Polygene (Rümlang, Switzerland) using the C57Bl/6-derived embryonic stem cell targeting. To obtain *Mut*$^{ko/ki}$ mice, females *Mut*$^{ko/wt}$ were crossed to *Mut*$^{ki/ki}$ males as previously described (Forny, 2016). Mouse genotyping was performed on genomic DNA from ear punch biopsies using the primers 5′-GTGGGTGTCAGCACACTTG-3′ (forward) and 5′-CGTATGACTGGGATGCCT-3′ (reverse) for the KI allele and 5′-ACAACTCCTTGTGTAGGTC-3′ (forward) and 5′-CCTTTAGGATGTCATTCTG-3′(reverse) for the KO allele. For mouse studies, no specific randomization or blinding protocol was used, the females (16-18 weeks) of both *Mut*$^{wt/wt}$ and *Mut*$^{ko/ki}$ mice were used, and all the experiments were performed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and with the approval of the Committee for Animal Rights of the University of Zurich (Zurich, Switzerland).

Metabolic cage studies and metabolic parameters. Long term monitoring of mice entailed weekly weight measurements and regular blood collections, as well as urine collections. The animals were single caged overnight to collect urine and measure individual chow and water intake; the urine was collected over ice in the morning and the diuresis was measured (Raggi, 2014; Luciani, 2016). Ammonia levels in whole blood were measured by the PocketChem blood ammonia meter (PA-4140; Arkray). The urine parameters were measured using a UniCel DxC 800 pro Synchron (Beckman Coulter, Fullerton, CA, USA), whereas MMA in mice urine was analyzed by liquid chromatography mass spectrometry (LC-MS/MS) on a Thermo Scientific UltiMate 3000 rapid separation LC coupled

to an AB Sciex 5500 TripleQuad mass spectrometer using a commercial kit (Recipe ClinMass® advanced).

***Kidney isolation and primary cultures of mouse proximal tubule cells*.** The kidneys were harvested from *Mut*$^{flox/flox}$ mice as previously described (Raggi, 2014; Luciani, 2016). One kidney was split transversally and one half was fixed and processed for immunostaining while the other half was flash-frozen, homogenized by Dounce homogenizer in 1 mL of RIPA buffer that contains protease and phosphatase inhibitors and processed for western blot analysis. The contralateral kidney was taken to generate primary cultures of mPTCs as described (24). Freshly micro-dissected PT segments were seeded onto collagen-coated chamber slides (C7182, Sigma-Aldrich) and/or collagen-coated 6- or 24-well plates (145380 or 142475, Thermo Fisher Scientific), and cultured at 37°C and 5% CO_2 in DMEM/F12 (21041-025, Thermo Fisher Scientific) with 0.5% dialyzed FBS, 15mM HEPES (H0887, Sigma-Aldrich), 0.55 mM sodium pyruvate (P2256, Sigma-Aldrich), 0.1ml/L non-essential amino acids (M7145, Sigma-Aldrich), hydrocortisone, human EGF, epinephrine, insulin, triiodothyronine, TF, and gentamicin/amphotericin (Single Quots® kit, CC-4127, Lonza), pH 7.40, 325 mOsm/kg. The medium was replaced every 48 h. Confluent monolayers of mPTCs were expanded from the tubular fragments after 6–7 days, characterized by a high endocytic uptake capacity. All experiments were performed on confluent monolayers grown on chamber slides or plates.

***Adenovirus transduction*.** For expression studies, adenovirus constructs used include CMV (control vector, Ad-CMV-GFP, Vector Biolabs) or an individually carrying Cre-recombinase (Ad-Cre-GFP, Vector Biolabs). Briefly, the cells were plated onto collagen-coated chamber slides or 24-well or 6-well tissue culture plates. Adenovirus transduction was performed 24h after plating when the cells reach approximately 70-80% confluence. The cells were subsequently incubated for overnight at 37°C with culture medium containing the virus at the appropriate concentration (0.2125×10^9 PFU/mL). The cells were afterwards provided with fresh medium every 2 days, cultured for 8 days and collected for analyses. Transduction efficiency was determined by viral GFP expression in live cells. GFP expression indicated that nearly 100% of cells were transduced after 8 days of transduction. The level of expression of mutase was determined by qPCR and proteins.

Quantitative real-time PCR.

Total RNA was extracted from organs of 10 weeks old female wild type mice of C57BL/6 background using Aurum™ Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, USA), according to the manufacturers' protocol. DNAse I treatment was performed to eliminate genomic DNA contamination. Total RNA was extracted from human urinary cells, mPTC and microdissected segments with RNAqueousR kit (Applied Biosystems Inc, USA), following the manufacturers protocol. Real-Time PCR was performed as described previously (Jouret, 2007; Bustin, 2009). The reverse transcriptase reaction was performed with 1 µg of RNA using iScript ™ cDNA Synthesis Kit (Bio Rad Laboratories, Hercules, CA). Changes in target genes mRNA levels were determined by relative RT-qPCR with a CFX96TM Real-Time PCR Detection System (Bio-Rad) using iQ™ SYBR Green Supermix (Bio Rad) to detect single PCR product accumulation. Specific primers were designed using Primer3 (Rozen, 2000) (Suppl. Tables 1 and 2) PCR conditions were 94°C for 3 min followed by 40 cycles of 15 sec at 95°C, 30 sec at 60°C. The PCR products were purified and sequenced using an ABI3100 capillary sequencer (Perkin Elmer Applied Biosystems). The efficiency of each set of primers was determined by dilution curves (Suppl. Tables 1 and 2). The relative changes in target over *GAPDH*/*Gapdh* mRNAs were calculated using the $2^{-\Delta\Delta}$Ct formula (Pfaffl, 2001).

Lysosomal activity. The detection of lysosomal activity was performed using Bodipy-FL-PepstatinA (P12271, Thermo Fischer Scientific) according to the manufacturer's specifications. Briefly, the cells were pulsed with 1µM Magic Red-$(RR)_2$ in Live Cell Imaging (A14291DJ, Thermo Fischer Scientific) medium for 1h at 37°C followed by fixation and immunostaining with anti-LAMP-1 or anti-HA antibody, and subsequently analyzed by confocal microscopy. (Luciani, 2016).

Extracellular Flux Analysis and Metabolic Assays.

OCRs and extracellular acidification rates (ECAR) were measured in a XF-96 Extracellular Flux Analyzers (Seahorse Bioscience) in cells suspended in XF medium (non-buffered RPMI 1640 containing either 25 mM glucose, 3,9 mM L-glutamine and 0,5 mM sodium pyruvate). Three

measurements were obtained under basal conditions and upon addition of oligomycin (2 µm), fluoro-carbonyl cyanide phenylhydrazone (FCCP; 0,5 µm), and rotenone (1 µm) + antimycin A (1 µm).

Mitochondrial membrane potential measurement. The mitochondrial membrane potential ($\Delta\psi_m$) was measured according to the manufacturer's specifications. Briefly, the cells were pulsed with 50nM Tetramethylrhodamine Methyl Ester Perchlorate (TMRM, T668 Thermo Fisher Scientific) for 30 min in live cell imaging at 37°C. After washing, the cells were subsequently analysed by confocal microscopy (Luciani, 2010).

ROS detection. The cells were pulsed with 5 µM CellROX® Green Reagent (C10444, Thermo Fisher Scientific) or with 2.5 µM MitoSOX Red Mitochondrial Superoxide Indicator for 10 min in live cell imaging at 37°C. After washing, the cells were subsequently analysed by confocal microscopy (Luciani, 2010).

Immunofluorescence and confocal microscopy

Human and mouse tissue kidneys: Fresh mouse kidneys were fixed by perfusion with 500-700 µl of 4% paraformaldehyde in PBS (158127, Sigma-Aldrich). The organs were dehydrated and embedded in paraffin at 58C°. Paraffin blocks of human and mouse kidney samples were sectioned into consecutive slices with a thickness of 6µm using a Leica RM2255 rotary microtome (Thermo-Fisher Scientific) on Superfrost Plus glass slides (12-550-15, Thermo-Fisher Scientific). Paraffin blocks were sectioned into consecutive 6µm-thick slices with a Leica RM2255 rotary microtome (Thermo-Fisher Scientific) on Superfrost Plus glass slides (Thermo-Fisher Scientific). Before staining, slides were deparaffinized in changes of CitriSolv (22-143-975, Thermo-Fisher Scientific) and 70% isopropanol. Antigen retrieval was accomplished by incubating in sodium citrate buffer (1.8% 0.1M citric acid, 8.2% 0.1M sodium citrate, in distillated water, pH 6.0) in a rice cooker for 30 minutes. The slides were quenched with 50 mM NH4Cl, blocked with 0.5% BSA in PBS Ca/Mg (D1283, Sigma-Aldrich) for 30 min and stained with primary antibodies specific for LC3 and p62/SQSTM1 diluted in blocking buffer overnight at 4°C. After two washes in 0.1% Tween 20 (v/v in PBS), the slides were incubated with corresponding fluorophore-conjugated Alexa secondary antibodies (Invitrogen) diluted in blocking buffer at room

temperature for 1 h and counterstained with 1µg Biotinylated Lotus Tetragonolobus Lectin (LTL) (B-1325 Vector Laboratories; marker of the PT apical membrane) and 10 µM Hoechst 33342 (H1399 Thermo Fischer Scientific). The slides were mounted in Prolong Gold Anti-fade reagent (P36930, Thermo Fisher Scientific), acquired on Leica SP8 confocal laser scanning microscope (Center for Microscopy and Image Analysis, University of Zurich) equipped with a Leica APO 63x NA 1.4 oil immersion objective at a definition of 1,024 x 1,024 pixels (average of eight or sixteen scans), adjusting the pinhole diameter to 1 Airy unit for each emission channel to have all of the intensity values between 1 and 254 (linear range). The micrographs were processed with Adobe Photoshop (version CS5, Adobe System Inc., San Jose, USA) software. Quantitative image analysis was performed by selecting randomly ~5 visual fields per each slide that included at least 3-5 PTs (LTL-positive), using the same setting parameters (i.e. pinhole, laser power, and offset gain and detector amplification below pixel saturation). The numbers of LC3 or p62/SQSTM1 structures were counted per field in the kidneys by two people (blinded to the experimental groups) and the average of their scoring was used for each micrograph.

Cultured cells. The cells were fixed for 10 min with 4% paraformaldehyde in PBS, quenched with 50 mM NH_4Cl and permeabilized for 30 min in blocking buffer solution containing 0.1% Triton X-100 and 0.5% BSA dissolved in PBS. Subsequently, the cells were incubated overnight with the appropriate primary antibodies at 4°C. After repeated washing with PBS, the slides were incubated for 30 min with the suitable fluorophore-conjugated Alexa secondary antibodies (Invitrogen), mounted with the Prolong Gold Anti-fade reagent, and analyzed by using a Leica SP8 confocal laser scanning microscope (Center for Microscopy and Image Analysis, University of Zurich) using the settings described above. Quantitative image analysis was performed by selecting randomly 3 visual fields pooled from biological triplicates, with each field including at least 10-15 cells, using the same setting parameters (i.e. pinhole, laser power, and offset gain and detector amplification below pixel saturation). The quantitative cell image analyses were performed using the open-source cell image analysis software CellProfiler™ (Carpenter, 2006). In particular, the pipeline “Speckle counting” was used to identify smaller objects (LC3 or SQSTM1 positive structures) surrounding larger objects and

to perform per-object aggregate measurements. The pipeline "Cell/particle counting, and scoring the percentage of stained objects" was used to score either the numbers of LC3/TOMM20 or TOMM20/PINK1 positive structures per cell.

Transmission electron microscopy. Mouse kidney sections and cells were fixed in 4% paraformaldehyde/0.1% glutaraldehyde in 100 nM sodium cacodylate, at pH=7.43, dehydrated and embedded in LR-White resin (LADD Research Industries). The grids were viewed on a Philips CM100 transmission electron microscope at 80kV.

Western blotting. Immunoblotting was performed as described previously (Raggi, 2014; Luciani, 2016). Proteins were extracted from isolated kidneys or primary cultured cells, lysed in lysis buffer containing protease (1836153001, Roche) and phosphatase inhibitors (04906845001, PhosSTOP Sigma), followed by sonication and centrifugation at 12,000 rpm for 10 min at 4°C. The samples were thawed on ice, normalized for protein (20µg/lane), dissolved in Laemmli sample buffer and separated by SDS–PAGE in reducing conditions. After blotting onto PVDF and blocking with 5% non-fat milk (1706404, Bio-Rad Laboratories) diluted in PBS, the membranes were incubated overnight at 4°C with primary antibody, washed, incubated with peroxidase-labeled secondary antibody, and visualized with enhanced chemiluminescence (WBKLS0050, Millipore, Life technologies). For re-probing, the membranes were rinsed, incubated for 30 min at 55°C in a stripping buffer (62.5mmol/L Tris-HCl, 2% SDS, 100mM mercaptoethanol, adjusted to pH =7.4), before incubation with primary antibodies. Quantitative analysis was performed by scanning the blots and measuring the relative density of each band normalized to β-actin, GAPDH or α- tubulin and using ImageJ software.

Statistical analysis. The quantitative data were expressed as means ± standard error of the mean (SEM). Differences between experimental groups were evaluated using analysis of variance followed by Bonferroni *post hoc* test, when appropriate. When only two groups were compared, unpaired or paired two tailed Student's *t*-tests were used as appropriate. The sample size of each experimental group is described in figure legends. The results are representative of at least three independent

experiments. GraphPad Prism software was used for all statistical analyses. Statistical significance was set at a $P<0.05$.

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6. Supplementary tables

Suppl. Table 1. Human primer pairs for gene expression analysis.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	PCR products (bps)	Efficiency
GAPDH	GGGGCTCTCCAGAACATCAT	TCTAGACGGCAGGTCAGGT	162	0.99 ± 0.02
MUT	CAATGGCAGCAGTATTTGGA	GATCAGCCACTTTGGGAATC	150	0.99 ± 0.03
NPHS2	TGGGGAATCAAAGTGGAGAG	CCTCAGGGACTCAGAAGCAG	153	1.01 ± 0.03
AQP1	GGACACCTCCTGGCTATTGA	GGCCAGGATGAAGTCGTAGA	162	1.02 ± 0.02
AQP2	GAAGCTCTTCCTGACACTG	TAGAGCAGCCGGTGTAATGG	149	1.03 ± 0.03
KCNJ1	CCAAGTCCGGACATCCTATG	CAGTGAGGGGTCTCCACTTC	141	0.99 ± 0.02
SLC38A3	AAGGACCCCTCCAAGAAGAA	TCCACCTTGCTGTAGGTGTG	149	0.98 ± 0.03
UMOD	CGAGTGTCACCTGGCGTAC	TGTGCTCCAGGAGGGAGATA	155	1.02 ± 0.03
SQSTM1	TGTGGGGAAGATTCTTGGAC	AGTGGGCATATTTGGGGTCT	152	1.01 ± 0.03
PCNA	ACGTCTCTTTGGTGCAGCTC	GCGTTATCTTCGGCCCTTAG	157	0.97 ± 0.03

Suppl. Table 2. Mouse primer pairs for gene expression analysis.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	PCR products (bps)	Efficiency
Gapdh	TGCACCACCAACTGCTTAGC	GGATGCAGGGATGATGTTCT	176	1.04 ± 0.03
Pink1	ATATGCTGCCCCCACACTAC	CAACTGCAAGGTCATCATGG	149	1.02 ± 0.04
Park2	TTGACACGAGTGGACCTGAG	GGGCCTTTGCAGTAGATGAA	152	0.98 ± 0.03
Npsh2	GTCTAGCCCATGTGTCCAAA	CCACTTTGATGCCCCAAATA	162	1.03 ± 0.03
Slc12a3	CATGGTCTCCTTTGCCAACT	TGCCAAAGAAGCTACCATCA	148	1.01 ± 0.03
Slc12a1	CCGTGGCCTACATAGGTGTT	GGCTCGTGTTGACATCTTGA	154	0.99 ± 0.04
Aqp2	TCACTGGGTCTTCTGGATCG	CGTTCCTCCCAGTCAGTGT	147	1.03 ± 0.04
Slc5a2	TTGGGCATCACCATGATTTA	GCTCCCAGGTATTTGTCGAA	164	1.01 ± 0.03
Slc38a3	GTTATCTTCGCCCCCAACAT	TGGGCATGATTCGGAAGTAG	109	0.99 ± 0.02

7. Supplementary Figures

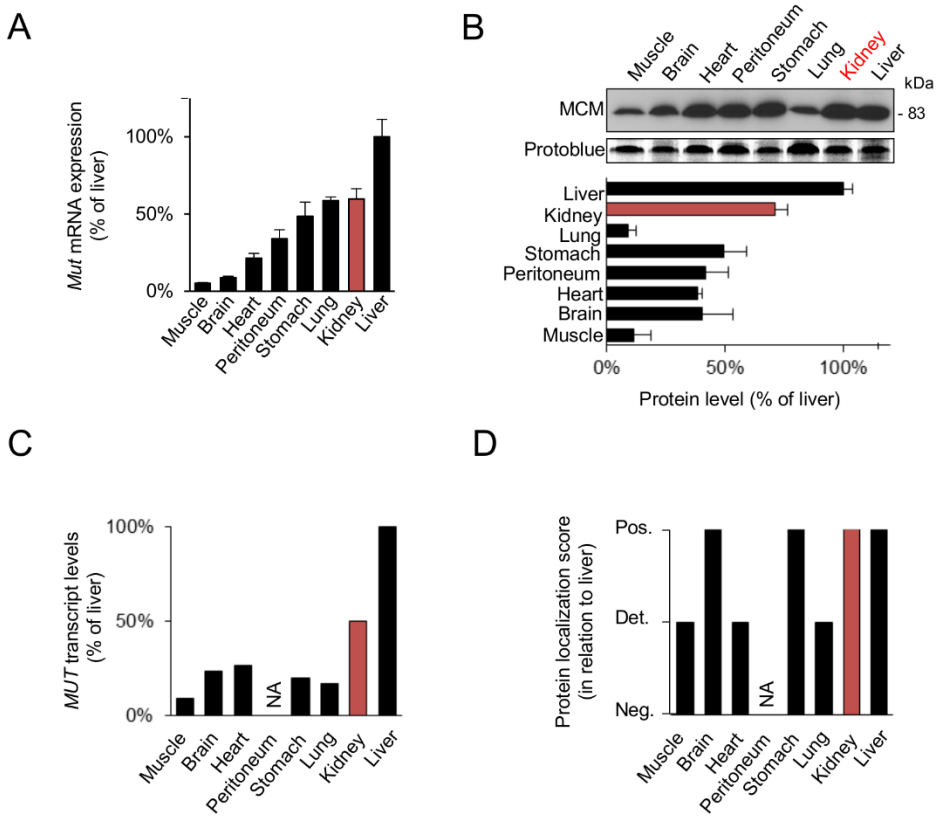

Fig. S1. Tissue expression of MUT in mouse and human. (A) Gene (Mut) and **(B)** protein (MUT) expression in different whole tissue lysates obtained from mouse by RT-qPCR and immunoblotting analyses. The graphs represent mean ± SEM, n=4. The gels were stained overnight with Protoblue. Data are normalized to those obtained from liver. **(C)** Expression profile of MUT in human tissues using RT-qPCR. The graphs represent mean ± SEM. **(D)** Protein levels of MUT in human tissues **((C), (D)** (Data compiled from the Human Protein Atlas (www.proteinatlas.org).)).

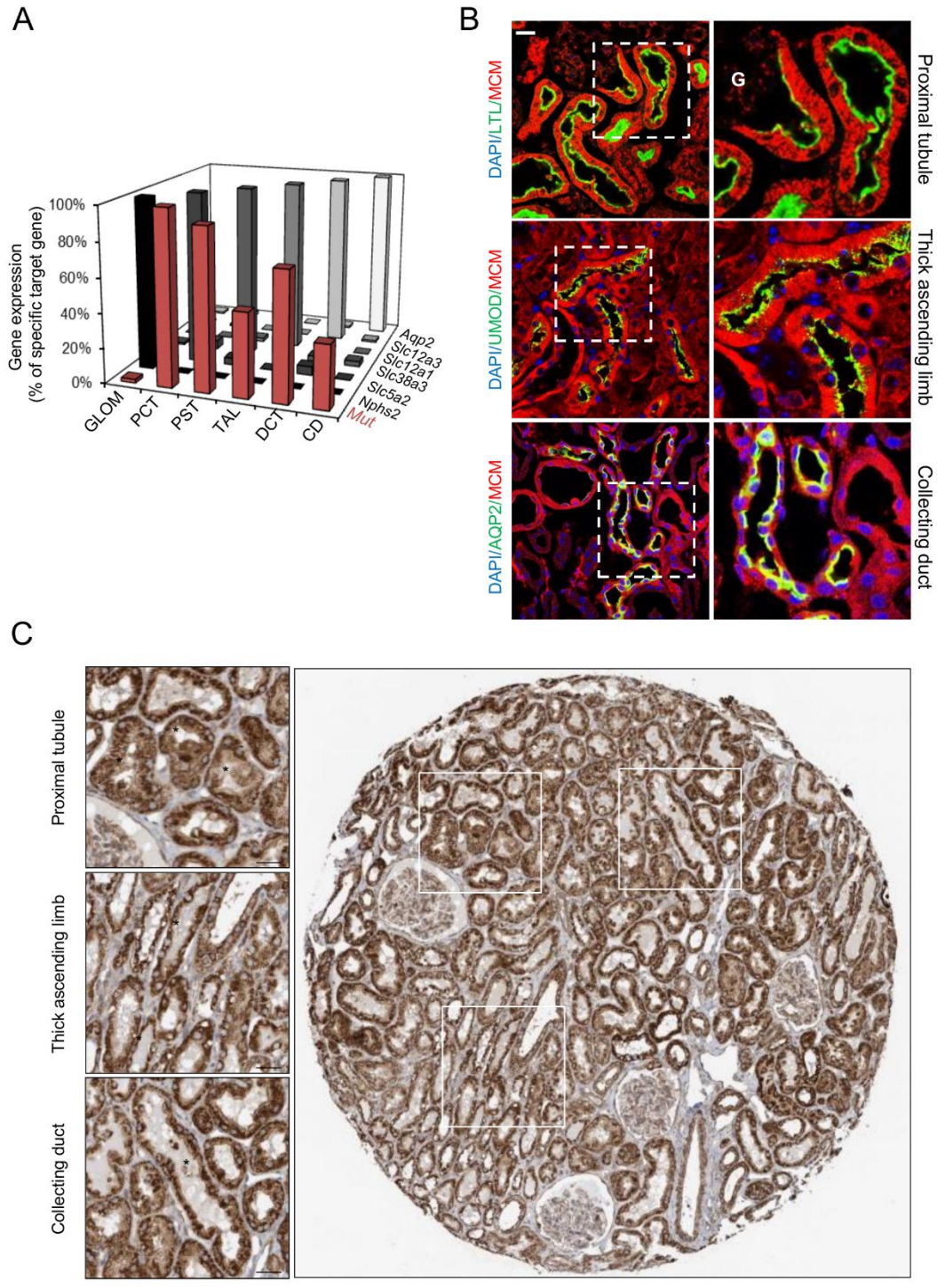

Fig. S2. Segmental expression of MUT in mouse kidney. (A) Expression profile of Mut in kidney segments by using RT-qPCR. Segment specific markers (e.g. Nphs2 for glomerulus (GLOM); Scl5a2 or Slc38a3 for proximal convoluted (PCT) or proximal straight tubule (PST), respectively; Slc12a1 for thick ascending limb (TAL); Slc12a3 for distal convoluted tubule (DCT) and Aqp2 for collecting duct (CD)) were used to score the purity of each tubule segment fractions. The graphs represent mean ± SEM, n=4. **(B)** Representative confocal micrographs of MUT in LTL (green, left)-positive proximal tubules or uromodulin-positive thick ascending limb (green, middle) or in aquaporinn-2 positive collecting duct segments (green, right panel) of mouse kidneys. The nuclei counterstained with DAPI (blue). Scale bars, 30µm. (C) Distribution of MUT in various tubular segments of the human kidney. (Data compiled from the Human Protein Atlas (www.proteinatlas.org).)

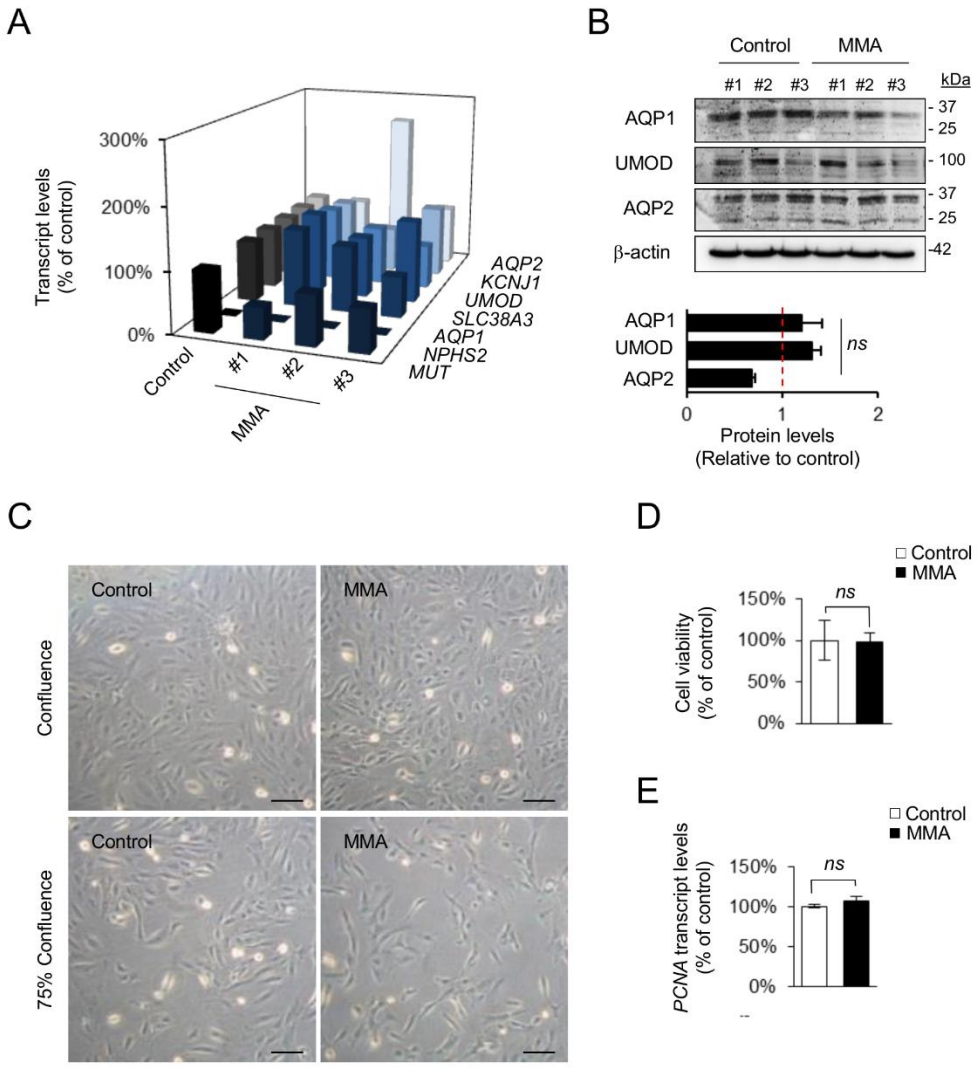

Fig. S3. Characterization of renal tubular cells derived from control and MMA patients. (A) Gene expression profile of *MUT*, *NPSH2*, *AQP1*, *SLC83A3*, *UMOD*, *KCNJ1* and *AQP2* in control and MMA patient-derived cells by RT-qPCR. (**B**) Western blotting and densitometric analyses of AQP1, UMOD and AQP2 protein levels in control and MMA cells. β-actin was used as a loading control. (**C**) Morphology of control and MMA cells was assessed by phase-contrast microscopy. Scale bar, 200 µm (**D**) Cellular viability and (**E**) *PCNA* gene expression of control and MMA cells monitored by Cell Counting Kit-8 assay and by RT-qPCR, respectively. Mean ± SEM; n=3, *ns*, not significant.

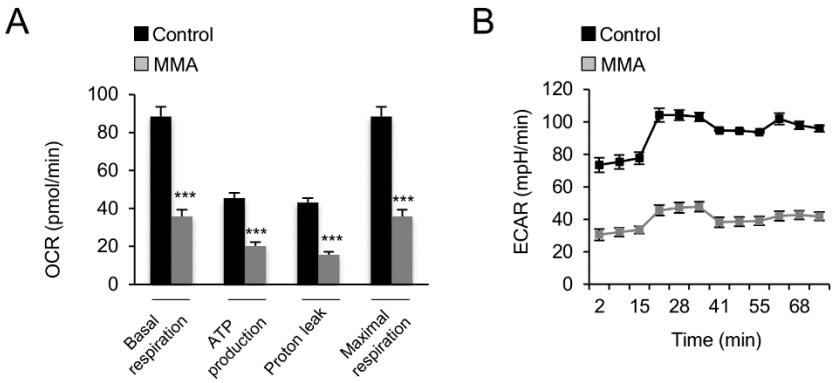

Fig. S4. Mitochondrial respiration is impaired in renal tubular cells derived from MMA patients. (**A**) Averaged values of different domains of mitochondrial function in control and MMA patientderived renal tubular cells. Bars are mean ± SEM. ***P<0.001, n=6 measurements per condition. (**B**) E t Extracellular acidification rates (ECAR) were measured in real time in control and MMA cells. The graphs represent mean ± SEM, n=6 measurements per each condition.

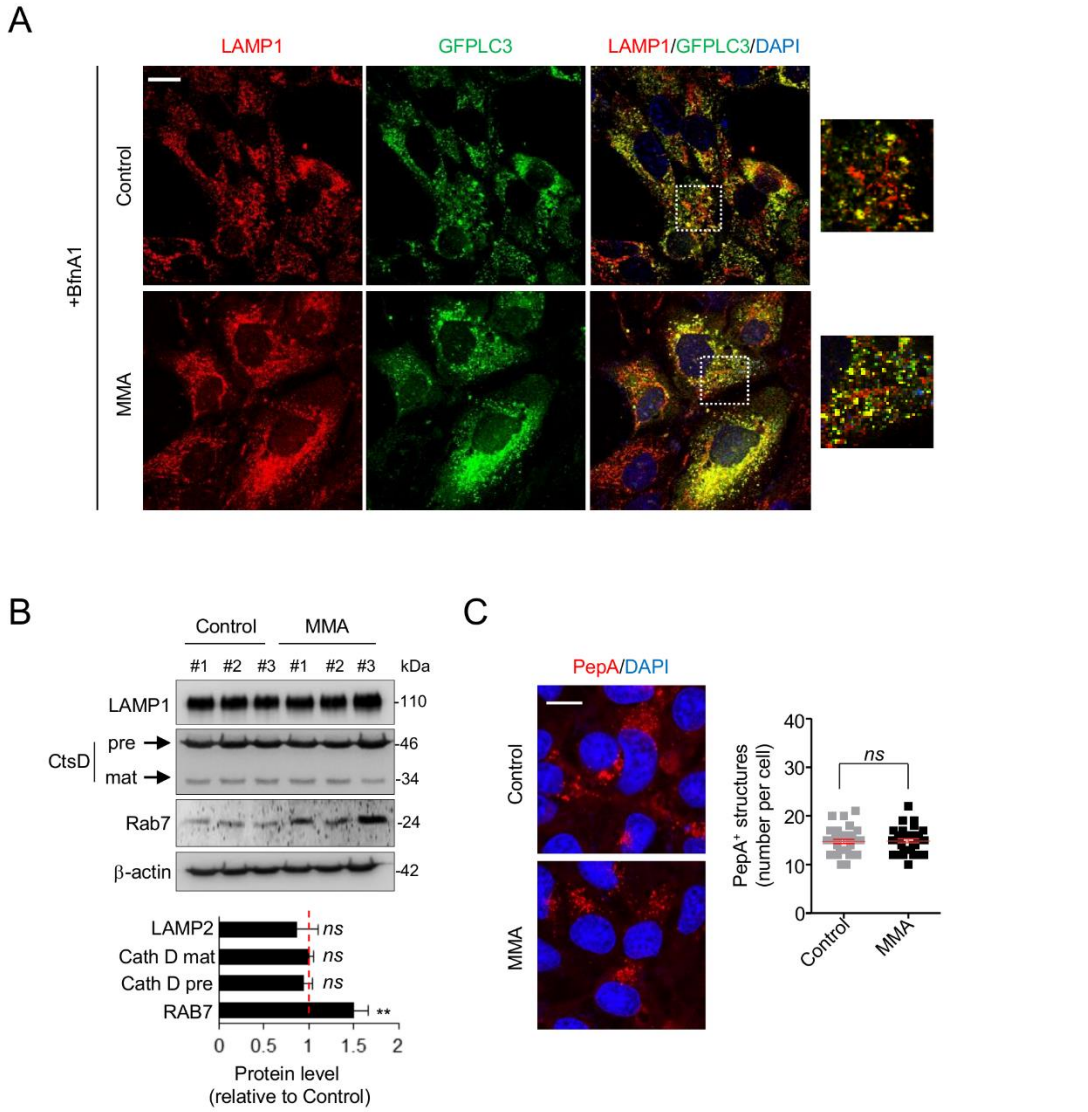

Fig. S5. Autophagosome-lysosome fusion and lysosomal degradation capacity are not altered in renal tubular cells from MMA patients. (**A**) The cells were transduced with GFP/mMAP1LC3B bearing adenoviral particles and cultured in presence of BfnA1 (50 nM for 1h), fixed and immunostained with anti-LAMP1 antibody, and analysed by confocal microscopy. Yellow indicates the colocalization. Nuclei are counterstained with DAPI (blue). Scale bar, 10µm. (**B**) Western blotting and densitometric analyses of LAMP2, Cathepsin-D and RAB7 protein levels in control and MMA cells. β-actin was used as a loading control. $^{**}P<0.01$ relative to control cells ($n=3$); *ns*, not significant. (**C**) The cells were loaded with MR-CtsB peptide (1mM, for 1h), fixed and analysed by confocal microscopy. Quantifications of MR-CtsB structures (n=30 randomly selected cells, each point representing the average number of in a cell; graphs represent mean ± SEM (n=3; *ns*, not significant). Nuclei are counterstained with DAPI (blue). Scale bar, 10µm.

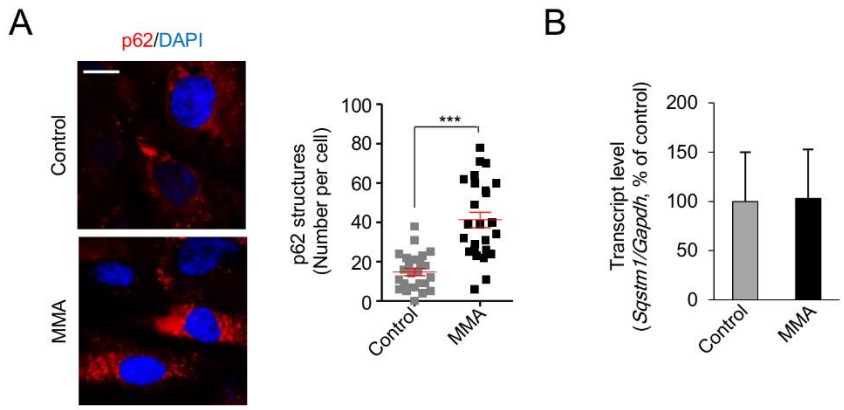

Fig. S6. Autophagy is dysregulated in renal tubular cells derived from MMA patients. (A) Representative confocal micrographs (left) and quantification of numbers of p62 structures (red) in MMA and control cells (n=30 cells pooled from three control and MMA cells; each point representing the average number of p62+ structures in a cell; graphs represent mean ± SEM). ***P<0.001 relative to control cells. Scale bar, 10 μm. **(B)** The mRNA levels of Sqstm1 were analysed by real-time PCR in controls (n=3) and in MMAcells (n=3). Bars are mean ± SEM. ns, not significant.

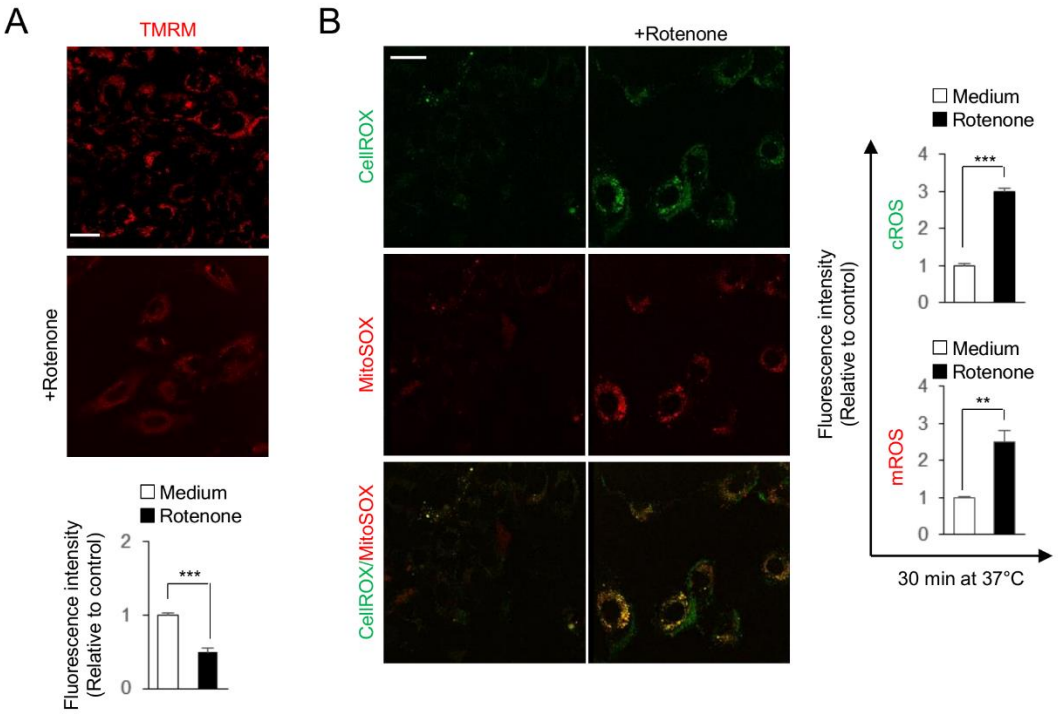

Fig. S7. Damaged mitochondria and mitochondrial oxidative stress in control cells exposed to Rotenone. (A-B) The cells were cultured in presence or in absence of Rotenone (10mM for 16 h). (A) The cells were loaded with tetramethylrhodamine methyl ester (TMRM; 50nM for 30 min) and analysed by live confocal microscopy. Quantifications of TMRM fluorescence intensity obtained from 3 randomly selected fields per condition, with each containing ~10-15 cells. ***P<0.01 relative to untreatedcontrol cells. **(B)** Cells were loaded with CellROX (cellular ROS probe; 5 mM for 10 min) and MitoSOX (mitochondrial ROS probe; 2.5 mM for 10 min) and analysed by live confocal microscopy. Quantifications of CellROX or MitoSOX fluorescence intensity was obtained from 3 randomly selected fields per condition, with each containing ~10-15 cells. ***P*<0.01 relative to untreated control cells.

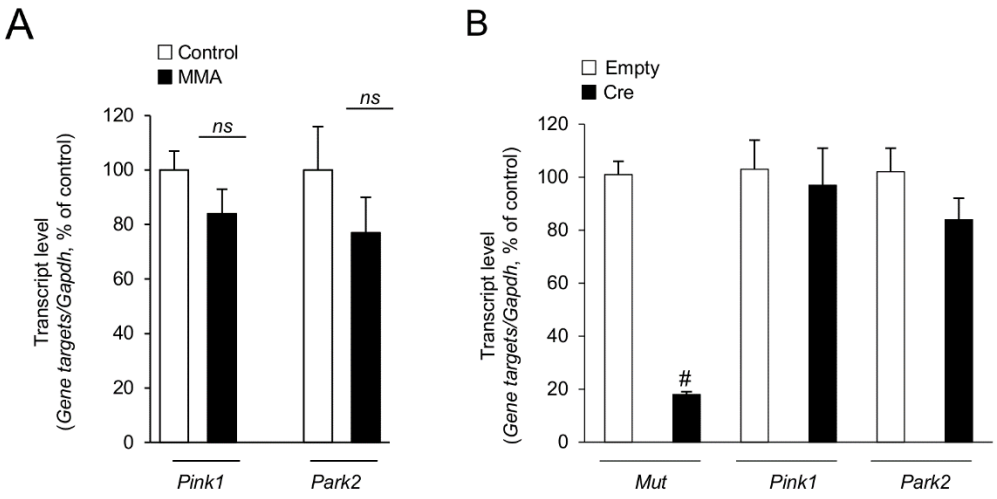

Fig.S8. The mRNA levels of *Mut*, *Pink1* and *Park2* were analysed by real-time PCR. The graphs represent mean ± SEM. (n=4 independent experiments). **(A)** Gene expression in control *vs.* MMA patient-derived cells; ns, not significant. **(B)** Gene expression in Empty *vs. Mut*-deleted mPTC. $^{\#}P$=5,52×10^{-6} relative to cells transduced with empty adenoviral particles.

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Conflict of interest statement: The authors state that they do not have any conflict of interest.

V. DISCUSSION AND PERSPECTIVES

This thesis investigated the influence of MUT deficiency leading to MMA and its impact on renal disease using a multi-level approach based on *in vitro* and *in vivo* models of human (renal tubular epithelial cell lines, kidney tissue) and murine (*Mut*$^{ki/ko}$ mouse model, mPTC cells from the *Mut*$^{flox/flox}$ mouse model) origin. Our data indicate the mitochondrion as key disease-driving organelle in MMA. Due to defective PINK1/parkin-mediated mitophagy, the autophagic removal of dysfunctional mitochondria is impaired. This results in accumulation of autophagic products and mitochondrial ROS causing cellular damage. Mitochondria-targeted anti-oxidant treatment restored mitochondrial integrity and homeostasis, rescued the mitophagy machinery and promoted clearance of dysfunctional mitochondria by autophagic degradation thus revising the cellular damage process. The data provide the mechanism linking mitochondrial dysfunction and renal epithelial cell damage and highlight the importance of PINK1/parkin-mediated clearance for mitochondrial degradation and renal epithelial homeostasis. Targeted mitochondrial treatment with anti-oxidant provides a novel strategy to treat renal tubular dysfunction in mitochondrial disorders such as MMA.

The epithelial cells lining the kidney tubules are particularly active in terms of active transport. Primarily the PT (Hall, 2013) and TAL (Emma, 2016) segments are involved in the reabsorption of essential nutrients and solutes and therefore need mitochondria to provide energy for ATP-consuming transport-mechanisms mainly provided by the basolateral Na^+/K^+-ATP-ase. Mitochondria are not only extremly vulnerable to ROS, but also a main source of ROS production. Un-balanced mitochondrial ROS levels will have a direct negative influence on renal cellular and kidney function.

Recent studies have shown that MMA leads to deficiency of the respiratory chain (Morath, 2008) and highly elevated ROS levels (Manoli, 2013) point at mitochondrial dysfunction to be involved in the onset and progression of kidney disease in MMA (Manoli, 2013). Patients present with renal tubular dysfunction and finally develop CKD due to tubulointerstitial lesions as evidenced by bioptic investigations (Haarmann, 2013). MUT is expressed in every tissue and organ, but the kidney is - besides the brain- the only organ suffering from early and chronic long-term complications. Hardly any organ specific studies have been conducted so far for a lack of appropriate models and tools. The investigation of tissue specific alterations caused by MUT deficiency in novel *in vitro* but also *in vivo* models is challenging and crucial to unravel disease associated mechanisms. This might lead to a better understanding of the underlying pathomechanisms causing CKD in MMA and reveal new treatment options.

In the first part of the thesis we illustrated the long-term follow up of a patient suffering from a vitamin B_{12} responsive CblA type MMA-uria due to the relatively common p.R145X nonsense mutation. Despite metabolic stability and normal development and growth, the patient developed CKD at the age of 12 years. She was re-tested at this time for vitamin B_{12} responsiveness. Of note, kidney

function could be stabilized by the administration of vitamin B_{12} for a period of 17 years before she had to undergo renal replacement therapy and finally kidney transplantation. This study highlights the importance of early and adequate vitamin B_{12} supplementation in responsive patients and underlines, that renal failure is the consequence even in relatively mild forms of MMA with excellent metabolic stability.

High concentrations of methylmalonic acid in the urine are a known risk factor for early and rapid development of CKD in MMA (Hörster, 2007). Regular application of high doses of vitamin B_{12} were able to reduce significantly the urinary excretion of MMA in our patient and probably prolonged the progression from CKD III to end stage renal disease for 17 years. After renal transplantation, plasma and urinary methylmalonic acid levels dropped pointing at the high MUT activity of ~20% provided by the transplanted kidney (van Calcar, 1998; Morath, 2008).

Several kidney biopsies were performed showing impressive tubular atrophy and interstitial fibrosis interspersed with mononuclear infiltrates. Despite the biopsies reflect different states of renal insufficiency in the patient's kidney, no MMA specific pattern could be detected, which fits well to previous reports (Lubrano, 2007). 6 years after kidney transplantation the graft biopsy did not reveal any striking abnormalities or hints at a relapse of kidney disease.

However, organ transplantation in MMA has to be interpreted with care: The limited amount of transplantations (kidney, liver or combined liver kidney) conducted were performed in a phenotypically highly heterogeneous group, most of them suffering from a mut(0) defect (Kaplan, 2006; Brassier, 2013; Kashahara, 2006). Although organ transplantation was able to improve metabolic stability in some patients, others were facing severe metabolic crisis, metabolic-stroke like episodes and death. A Japanese group (Sakamoto, 2016) recently underlined the importance of liver transplantation for metabolic stability in a cohort of MMA patients for growth and metabolic stability but clearly states that transplantation is neither a cure nor allows following a normal diet. The kidney seems to play a key role in the context of MMA: Patients receiving a kidney or a combined kidney liver transplant had lower levels of MMA and a better metabolic stability (Brassier, 2013; Kashahara, 2006) than compared to liver only transplanted patients.

In conclusion, the long-term follow up clearly underlines that CKD is a long-term consequence even in (almost) optimal metabolic conditions with only few and mild metabolic derailments. Vitamin B_{12} treatment significantly lowered MMA levels and CKD progression and should therefore be carefully evaluated in every MMA patient. Organ transplantation and kidney transplantation in particular can give metabolic stability but does not cure MMA nor solve the dietary issue.

For this reason, it is obvious that an in-depth investigation of the causal pathophysiology of CKD is crucial for a better understanding of mechanisms induced by MMA. This might in the long run lead to novel, more targeted treatment options.

In the second part of the thesis we investigated the subcellular changes leading to renal cellular damage in MMA *in vitro* and traced back key findings in the novel *Mut*$^{ki/ko}$ mouse model and a human kidney biopsy *in vivo*. The aim was to provide multilevel evidence that disruption of the *MUT* gene is associated with changes in the dynamic equilibrium of renal mitochondrial metabolism and the pathway leading to epithelial damage. We showed in different *in vitro* (human renal tubular epithelial cell lines, Cre mediated *Mut* deletion in mPTC from *Mut*$^{flox/flox}$ mice) and *in vivo* (*Mut*$^{ki/ko}$ mouse model, human kidney biopsy) systems that mitochondria are fragmented and dysfunctional in models lacking functional MUT. Dysregulation of the PINK1/parkin pathway causes impaired autophagic clearance of defective mitochondria resulting in increased autophagic flux with accumulation of autophagic products. Even after challenge with the mitochondrial toxin Rotenone the PINK1/parkin pathway remained down-regulated, indicating a defective mitochondrial targeting mechanism in MMA. Elevated markers for kidney damage suggested that these changes might be causal for CKD pointing at the importance of mitochondrial homeostasis in the pathogenesis of cellular damage in MMA. Cre mediated *Mut* deletion in mPTC from the *Mut*$^{flox/flox}$ mouse model was able to reproduce the observed findings underlining the importance of MUT activity for mitochondrial homeostasis. Treatment of human renal epithelial cells with mitochondria-targeted anti-oxidant therapy had a beneficial effect on the observed events. These results shed new light on the mechanisms leading to CKD in MMA and indicate that targeting mitochondrial ROS production restores mitochondrial homeostasis and reduces kidney damage.

MUT is expressed in all human tissues (Uhlén, 2015). Highest MUT activity is detected in the liver and the kidney followed by heart and brain (Wilkemeyer, 1997). Expression studies in female wild type mice of C57BL/6 background confirmed this data rendering mice a suitable model for *in vivo* studies. Microdissection of *Mut*$^{wt/wt}$ mouse kidneys revealed that MUT is expressed in all kidney segments with exception of the glomerulus. MUT deficiency might therefore not only affect specific kidney sections but the kidney as an entire organ.

To investigate the role of MUT and the impact of MUT deficiency on a subcellular, tissue specific level, we characterized immortalized cell lines derived from the urine of healthy controls and MMA patients harboring a *Mut*(0) mutation (Ruppert T, 2015). While expression of kidney specific markers of all tubular segments were comparably high in all cell lines, MUT activity was significantly reduced in MMA cells, rendering the cell lines a robust model.

MUT is a mitochondrial enzyme as indicated by its mitochondrial target-sequence (Fenton, 1982). Due to its energetic needs the kidney is rich in mitochondria which lead us to closely investigate this

organelle in the context of MMA. Our findings revealed remarkable alterations of mitochondrial morphology and function: MUT deficiency leads to decreased numbers of swollen, fragmented mitochondria with severe defects of their christae structure. Energetic profiling revealed a reduction of mitochondrial membrane potential as well as a reduction of baseline respiration, total respiration capacity and ATP turnover. The findings were paralleled by massive elevation of mitochondrial oxidative stress levels and renal epithelial damage in both MMA *in vitro* models. The findings could be confirmed in kidneys of the novel *Mut*$^{ki/ko}$ mouse model (Forny, 2016). These results are in line with Manoli et al. describing ultrastructural changes and respiratory chain dysfunction in a *Mut*$^{ko/ko}$ mouse model rescued from neonatal lethality by transgene expression in the liver (Manoli, 2013). Morath et al. (Morath, 2008) summarize the inhibition of mitochondrial energy metabolism by metabolites of alternative propionate oxidation leading to ROS generation in different *in vitro* settings and MMA patients' fibroblasts. These findings highlighted the mitochondrium as key organelle in promoting cellular damage in MMA. The mechanisms by which MUT deficiency disturbes mitochondrial homeostasis and how mitochondrial dysfunction is linked to cellular damage, remained, however, unclear.

Mitochondria are highly dynamic and metabolically active organelles. In renal tubular cells performing intense reabsorption and excretion processes at high energy costs, intact mitochondrial function is crucial for the maintenance of cellular health (Emma, 2016). Excess production of ROS leads to cellular dysfunction and tissue damage. Mitochondria themselves are a source of excessive ROS production as a side product of oxidative phosphorylation along the respiratory chain (Sena LA, 2012). To maintain mitochondrial integrity and homeostasis, MQC by the tightly inter-connected processes autophagy and mitophagy is essential (Ni, 2015; Kotiadis, 2014). Particularly in the kidney activation of autophagy has been associated with renal protective mechanisms, while pharmacological or genetic deletion of important autophagic proteins induced elevated ROS levels and cellular damage (Liu, 2014; Yamamoto, 2016).

In a next step, we investigated MQC and tested whether MUT deficiency leads to induction of altered autophagic flux in our different MMA models. Up-regulation of the autophagy machinery, evidenced by elevated levels of the autophagic proteins p62 and LC3-II going in hand with vast amounts of p62 and LC3-II positive vesicles could be detected in all *in vitro* and *in vivo* models. These findings were sustained by excessive accumulation of autophagic vacuole formation documented by EM investigation. Activation of the autophagy machinery has recently been described as a renal-protective mechanism in acute kidney injury by different groups (Isaka, 2011; Liu, 2014).

Autophagy is a tightly orchestrated process and accumulation of autophagic proteins can be induced at any step down-stream towards lysosomal degradation: Autophagosome formation and trafficking can be impaired as well as autolysosomal fusion or lysosomal degradation. Defective autolysosomal

fusion, e.g., is a hallmark of many lysosomal storage diseases (Ballabio, 2009). We tested this hypothesis by treating MMA cells with Bafilomycin (BfnAI), a drug accurately measuring autophagic flux (Bento, 2016). BfnAI treatment increased the levels of LC3-II in both control and MMA cells. Co-localization with the lysosomal marker LAMP-1 suggested undisturbed delivery of LC-3 positive cargo to the lysosome releaving that autolysosomal fusion is not compromised in MMA cells. The results pointed at either increased autophagosome synthesis or decreased lysosomal degradation capacity. The latter hypothesis could be ruled out since the lysosomal proteins LAMP2 and cathepsin D (CtsD) were present in equal amounts as compared to control cells and lysosomal activity measured by fluorescent tagged Pepstatin A binding active CtsD was comparably high in both groups. The fact that BfnAI treatment increased LC3-II levels in control and MMA cells strongly supports the hypothesis, that MUT deficiency indeed induces autophagosome synthesis rather than their clearance.

Autophagy plays a key role in cellular stress response. Increased LC3 fluxes in the MUT deficient models suffering from mitochondrial dysfunction underline the hypothesis that autophagy is crucial to counter-act stressful conditions. The accumulation of dysfunctional mitochondria and their toxic byproducts leads to potentiation of cellular stress and further impairment of cellular function (Twig, 2008). The selective removal of dysfunctional mitochondria (=mitophagy) is mediated by molecular mechanisms detecting potentially dangerous cargo and converting them into signals which lead to initiation of autophagic degradation. A well-described pathway for mammalian mitophagy is governed by the kinase PINK1 and the E3 ubiquitin ligase parkin whose close interaction allows the sensing and removal of dysfunctional mitochondria (Valente, 2004; Kitada, 1998). The importance of PINK1 and parkin for mitochondrial integrity and homeostasis is underlined by the fact that loss of either protein leads to mitochondrial dysfunction inducing muscular and neuronal dysfunction in drosophila models (Greene, 2003; Park, 2005). In healthy mitochondria, PINK1 is constantly cleaved from the OMM and degraded by several proteases (Greene, 2012). In stressful conditions leading to a loss of mitochondrial membrane potential, PINK1 is not longer processed but accumulates on the OMM recruiting parkin. This step allows the attachment of ubiquitin tags which label dysfunctional mitochondria for autophagic degradation (Valente, 2004). Rotenone is a well-known mitochondrial toxin inducing mitochondrial membrane depalorization. Treatment with Rotenone allowed us to track mitophagy events and concomitant autophagic sequestration. Indeed, Rotenone treatment led to accumulation of full length PINK1 on the OMM of dysfunctional mitochondria (counter-stained by TOMM20) and initiated autophagosomal degradation (as shown by co-localization of defective mitochondria (TOMM20) and LC-3 positive autophagosomes) in control cells. MMA cells were unable to react comparably to the rotenone stimulus depicting a lack of PINK1 and parkin already at baseline conditions and absence of PINK1 recruitment to the OMM after the rotenone challenge. Consequently, mitochondria of MUT-deficient models are not correctely labelled for autophagic degradation despite abundant expression of p62, a stress induced ubiquitin binding protein involved in the process of autophagosomal degradation (Cohen-Kaplan, 2016). Deficiency of the mitophagy

machinery might thus explain the defective clearance of dysfunctional mitochodnria and explain excessive accumulation of autophagic products.

Elevated levels of kidney damage markers (LCN2, KIM1) and disturbed renal function in the *Mut*$^{ki/ko}$ mouse model suggest a link between disturbed mitochondrial integrity and renal damage in MMA. Bueno et al (Bueno, 2014) recently related deficiency of PINK1/parkin mediated mitophagy and mitochondrial dysfunction to tissue damage and fibrosis induction in a pulmonary fibrosis model. Tubulointerstitial lesions transiting to fibrosis are frequently found in MMA patients' kidney biopsies underlining the importance of that finding (Rutledge, 1993; Goyenechea, 2012).

The question arising from the previous findings was whether the observed alterations *in vitro* and *in vivo* are related to MMA caused by monogenic MUT deficiency. To address this point, we performed Cre mediated *Mut* deletion of mPTC derived from a *Mut*$^{flox/flox}$ model. Cre mediated *Mut* deletion reproduced all observed findings including substantial changes of mitochondrial integrity and homeostasis, deficiency of the mitophagy machinery, accumulation of autophagic marker proteins and elevation of markers of kidney damage. These findings emphasize the role of MUT activity for mitochondrial homeostasis and link ROS and activation of autophagy to renal tubular damage. Elevation of LCN2 in the context of MUT deficiency plays a key role, since elevated LCN2 levels have been identified to play a disease driving role in CKD in mice and humans (El Karoui K, 2016; Viau, 2010).

Our findings suggested excessively elevated ROS levels to be a major complication in all MMA models. ROS is a well-known inducer of cellular and tissue damage. This is why we applied targeted anti-oxidant treatment with MitoTEMPO (MT) to control and MMA patients cell lines. Because of covalent binding of a lipophilic cation (triphenylphosphonium) to endogenous anti-oxidant CoQ_{10} the drug accumulates preferentially in the mitochondria, where it exerts a direct anti-oxidant reaction by neutralizing peroxyl, peroxynitrites and superoxide. Taking advantage of its oral bioavailability and targeted anti-oxidant effects, the efficiacy of MT treatment has been demonstrated in different disease models associated with excessive ROS production such as sepsis models, neurodegenerative disease and ischemia reperfusion injury (Jin, 2014).

MT treatment rescued mitochondrial morphology and function, restored mitochondrial mass levels and increased the ratio of uncleaved *vs.* cleaved PINK1 in MMA cells, allowing autophagic degradation as evidenced by reduced levels of autophagic proteins. Finally, LCN2 levels were markedly reduced in MMA cells pointing at a beneficial effect for cellular damage in treatment conditions. Altough MT treatment provided promising results in restoring mitochondrial homeostasis and cellular function further studies in animal models are necessary before translating these findings to the patient.

To conclude, MMA inevitably leads to CKD and end stage renal disease despite optimal metabolic management and cofactor supplementation, which is a major burden and live-time risk on all MMA patients. We identified in a multi-level approach the mitochondrion to be the key disease driving organelle in MMA. Mitochondrial integrity is severely hampered due to MUT deficiency. MQC is defective in MMA, as indicated by a highly active autophagy but deficient mitophagy machinery leading to a loss of mitochondrial homeostasis. These changes are paralleled by elevated LCN2 levels linking MUT deficiency to cellular and tissue damage. Targeted anti-oxidant treatment is able to reverse the above findings on a subcellular level highlighting the deleterious effect of excessive ROS production for disease progression. Further studies *in vitro* and *in vivo* will provide evidence whether anti-oxidant supplementation is a new therapeutic option to treat renal tubular dysfunction in MMA and other mitochondrial disorders.

Inborn errors of metabolism represent a monogenic group of defects with primary and secondary impacts on whole body metabolism. Characterization of metabolic changes and implications on human health and disease therefore are challenging. Understanding of the consequence of monogenic disease might be fundamental to draw conclusions for multifactorial, complex diseases in a next step. However, most of the studies on monogenic diseases like MMA have been narrowly focussed on the affected pathways neglecting involved pathways and possible targetable aims up- or down-stream. A main goal of physicians dealing with patients suffering from rare metabolic disease is to provide them with therapeutic interventions significantly improving their acute and chronic complications. As discussed for MMA, therapeutic interventions are limited and chronic complications a severe burden on MMA patients.

In this context, MMA as a monogenic disease is a prime candidate for an *in-silico* modeling approach. MMA qualifies in particular, since 1) the mitochondrial enzyme is highly expressed in the kidney, the most important target organ, 2) MUT does not have an impact on structural changes which might secondarily affect metabolic functions and 3) well-characterized *in vitro* and *in vivo* models of murine and human origin presented in this thesis allow a rapid and robust testing of the obtained hypothesis. In the aera of "omic" approaches, non-invasive investigation of human body fluids (e.g. blood, urine) by large sacle screening approaches (proteomics, metabolomics) can be used to confirm the relevance for affected individuals (Fig. 9). Algorithms to predict changes in metabolic flux and metabolites between controls and MUT deficient individuals will result in a ranked- list of prime candidates allowing for systematic and rapid evaluation as novel biomarkers or potentially targetable aims. Another important point is the identification of possible "by-pass" candidates whose activation might lead to a circumvention of the metabolic block. The efficiency and importance of *in silico* modeling in the metabolic field has recently been demonstrated by Pagliarini et al. (Pagliarini, 2016).

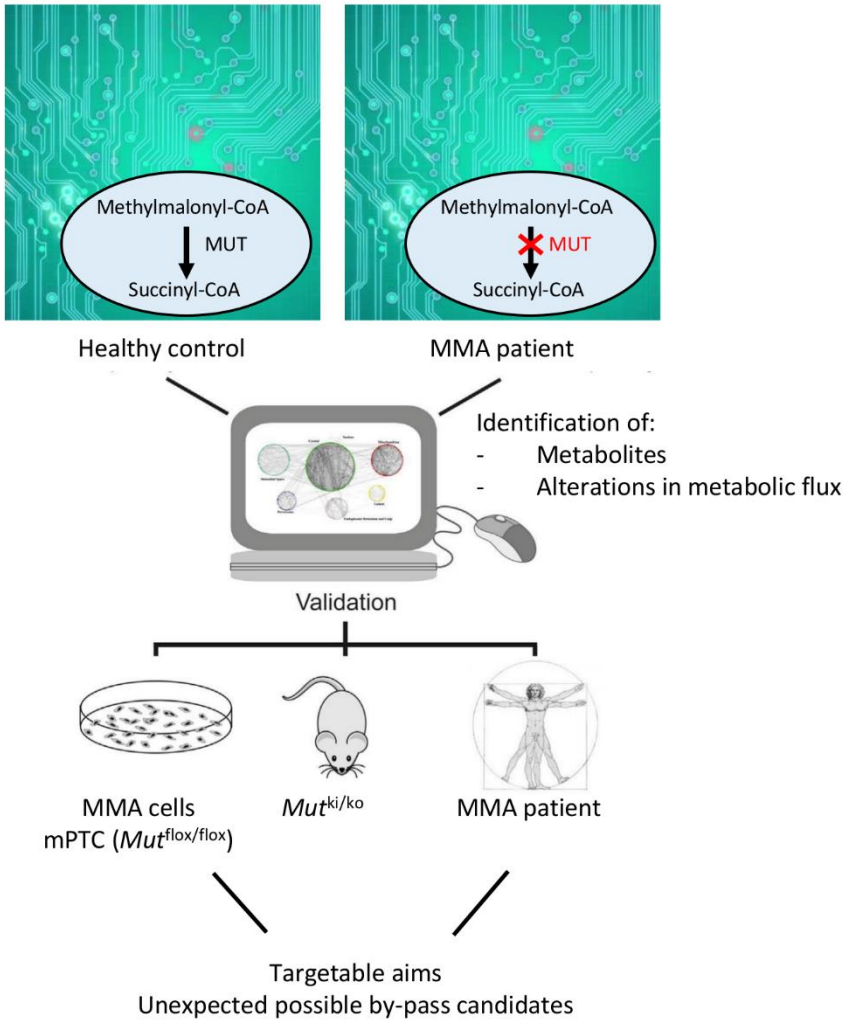

Figure 9: *In silico* model predicting alterations in metabolix flux caused by MUT deficiency. Loss of MUT activity is simulated in a computational model allowing to predict changes in metabolites and metabolix flux. Investigation of different *in vitro* and *in vivo* MMA models discussed in the thesis could be used for translation of the findings. Omic approaches of human body fluids will test the revevance of newly identified candidates. (Adapted from Pagliarini, 2016).

However, understanding of complex metabolic networks might only be one piece of the puzzle leading to new therapeutic options. New computational tools (Mode of Action by NeTwoRk Analysis=MANTRA, Iorio, 2016) are capable to analyze the mode of action of novel drugs and able to identifiy approved candidates for "drug-repositioning". The algorithm relies on a genome wide ranked expression list of genes sorted according to their expression after drug-treatment. To explore approved drugs to identify "repositionable-drugs" the system is ready to use. Based on our findings in this thesis, MANTRA could be used to predict the efficacy and possible side effects of treatment with e.g. autophagy inducers (e.g. Rapamycin), stimulators of mitochondrial biogenesis via PGC-1-α (fibrates, glitazones) or evaluate the advantages/disadvantages of targeted (MT) and un-targeted (e.g. N-acetylcysteine) anti-oxidant treatment. Exploration of the reference-drug neighbourhood might reveal more favourable candidates imposing less side effects.

This thesis demonstrates that MUT deficiency severly impacts mitochondrial homeostasis and that impaired PINK1 and parkin mediated clearance of dysfunctional mitochondria generate ROS leading to tubular cell damage. Targeting ROS to ameliorate kidney damage is the logical consequence. The advantage of targeting ROS is the fact that a large variety of different compounds acting at different aims (lipid membrane, cystosol, glutathione system, mitochondria) is already available on the market and FDA-approved (e.g. vitamin E, N-acetylycsteine, allopurinol, CoQ10) (Fig. 10, Small, 2012). However, the systemic effects as well as the advantages of targeted *vs.* untargeted treatment in the context of MMA have never been studied. The *Mut*$^{ki/ko}$ mouse model characterized by a renal phenotype will serve as a valid model to invatigate the effect of anti-oxidant treatment on kidney function in a next step before translation to bedside.

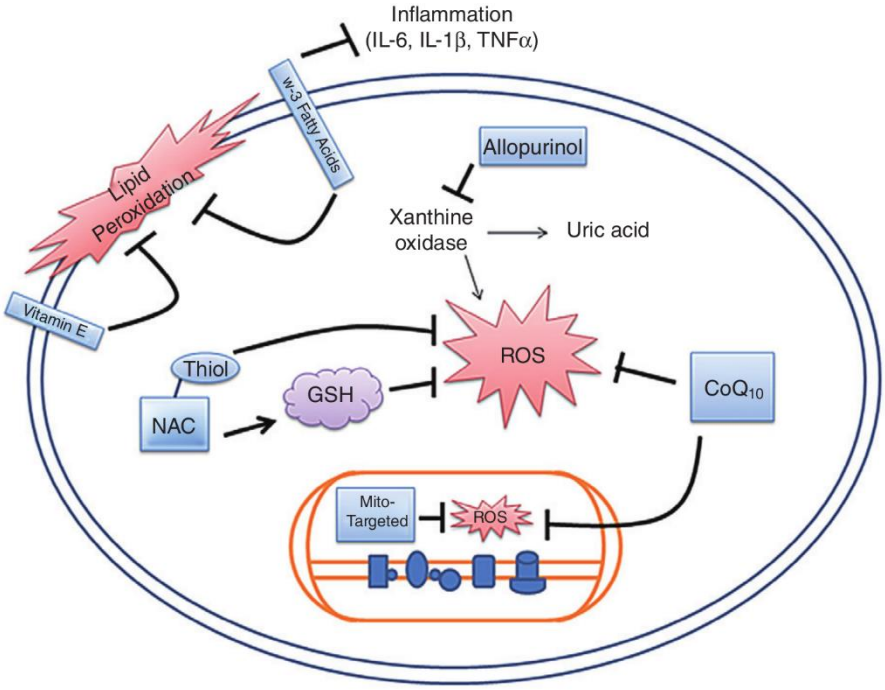

Figure 10: Cellular sites of ROS reduction mediated by different anti-oxidant compounds. Vitamin E acts on phospholipid bilayers. Allopurinol inhibits xanthine oxidase to reduce ROS production. N-acetylcysteine (NAC) acts on glutathione metabolism and CoQ_{10} enhances respiratory chian activity. (Adapted from Small, 2012).

However, studies in mice bear the disadvantage that breeding is costly and generation time is long compared to other models. The zebrafish has recently become a promising vertebrate disease model for phenotype based drug screening. Its genome shares 82% of human disease-causing proteins rendering the fish a suitable model organism. A reliable MMA zebrafish-model reproducing all metabolic hallmarks of MMA has recently been generated in the Devuyst group. The big advantage of the zebrafish would be that the assay output would provide a whole organism phenotype integrating multiple biochemical pathways. Unlike worms or flies, the zebrafish possesses organ systems

comparable to man (e.g. kidney) and organ function could be a direct read out. Phenotypic screens provide i) discovery of drugs in the absence of a validated target, ii) identification of compounds producing effects through activation of multiple targets and iii) identification of beneficial and unpleasant side effects in the same screen. The findings could be immediately applied to our *in vitro* and *in vivo* models and later translated to the patient.

The combination of highly focused tissue specific *in vitro* and *in vivo* studies on the one hand and highly predictive computational *in silico* models in combination with large-scale screening approaches will add substantial information to the understanding of MMA and its involvement in CKD onset and progression and might provide new therapeutic options.

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VII. CURRICULUM VITAE

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Born: 28.09.1979 in Bonn, Germany

Education and training

Academic studies:

1999-2006:	Medical school, Ruprecht-Karls-Universität Heidelberg
2001:	Preliminary medical examination
2002:	1st State examination
2005:	2nd State examination
2006:	3rd State examination, license to practice medicine

Practical year:

09/05-10/06: Department of Internal Medicine, University Hospital Heidelberg
Department of Surgery (General and Traumatology) Kantonsspital Uri, Switzerland
Institute of Human Genetics, University Hospital Heidelberg (Policlinic, Laboratory of Human Molecular Genetics)

Doctoral thesis:

05-07:	University Hospital Heidelberg, experimental doctoral thesis, Center for Pediatric and Adolescent Medicine, supervisor: Prof. Dr. B. Tönshoff
Thesis:	*The meaning of the insulin-like growth factor/insulin-like growth factor binding protein system for the differentiation of growth-plate chondrocytes. (title translated)*
11/07:	Granting of doctorate *(Dr. med.)*

Clinical education:

Center for Pediatric and Adolescent Medicine, University Hospital Heidelberg

Education sections:

01/07-10/07:	Division of Inborn Errors of Metabolism
10/07-04/08:	Division of Oncology, Hematology and Immunology
04/08-09/08:	Division of Neonatology
11/08-03/09:	Outpatient Policlinic and Emergency Department
07/09-10/09:	Division of Nephrology and Dialysis
10/09-03/11:	Intensive care unit (general, neonatal and transplantation intensive care medicine)
04/11-09/11:	Outpatient Policlinic and Emergency Department
10/11-05/12:	Evaluation of diagnostics and counseling in metabolic questions, Metabolic Laboratory, University Hospital Heidelberg
03/12:	Granting of *„Specialist in pediatrics"*
07/15:07/17	Fellow in the metabolic unit of the Children's University Hospital Zurich aiming for the *European certificate in pediatric metabolic medicine*, examination 03/18

Research education:

Division of Inborn Errors of Metabolism, research laboratory for basic science, University Hospital Heidelberg, supervisor: Prof. Dr. S. Kölker

09/08-10/08: *Transport of organic acids in renal proximal tubule epithelial cells under physiological conditions.*

04/09-06/09: *Influence of organic acid accumulation in methylmalonic aciduria: Altered transport and altered enzyme activity of the TCA cycle and the respiratory chain in renal proximal tubule epithelial cells.*

06/12-12/16: PhD thesis
University of Zurich, Institute of Physiology
"Mechanisms of inherited kidney disorders (MIKD)", Prof. Dr. O. Devuyst
University children's Hospital Zurich
Division of Metabolism, Prof. Dr. M. Baumgartner
Novel insights into the pathophysiology of kidney disease in methylmalonic aciduria.

Publications:

Schumann A*, Luciani A*, Berquez M, Tokonami N, Debaix H, Forny P, Kölker S, Diomedi Camassei F, CB, MK, Faresse N, Hall A, Ziegler U, Baumgartner M, Devuyst O.
Impaired Mitophagy Links Mitochondrial Dysfunction and Epithelial Cell Damage in Methylmalonic Aciduria. *(In preparation)*

1. Forny P, Schumann A, Mustedanagic M, Mathis D, Wulf MA, Nägele N, Langhans CD, Zhakupova A, Heeren J, Scheja L, Fingerhut R, Peters HL, Hornemann T, Thony B, Kölker S, Burda P, Froese DS, Devuyst O, Baumgartner MR. Novel Mouse Models of Methylmalonic Aciduria Recapitulate Phenotypic Traits with a Genetic Dosage Effect. J Biol Chem. 2016 Sep 23;291(39):20563-73. doi: 10.1074/jbc.M116.747717.

2. Ruppert T, Schumann A, Gröne HJ, Okun JG, Kölker S, Morath MA, Sauer SW. Molecular and biochemical alterations in tubular epithelial cells of patients with isolated methylmalonic aciduria. *Hum Mol Genet. 2015 Sep 29.*

3. Devuyst O., Schumann A. Peritoneal Dialysis: Nanoparticles have entered the game. (Editors' digest) *Peritoneal dialysis, Vol. 35, May 2015*

4. Haarmann A., Mayr M., Kölker S., Baumgartner E.R., Schnierda J., Hopfer H., Devuyst O., Baumgartner M.R. Renal involvement in a patient with cobalamin A type (cblA) methylmalonic aciduria: a 42-year follow-up. *Mol Genet Metab. 2013 Sep 17. doi:pii: S1096-7192(13)00317-X. 10.1016/j.ymgme.2013.08.021.(Cover story)*

5. Sauer SW, Opp S, Haarmann A, Okun JG, Kölker S, Morath MA (2009).
Long-term exposure of human proximal tubule cells to hydroxycobalamin (c-lactam) as a possible model to study renal disease in methylmalonic acidurias.
J Inherit Metab Dis. ***32*** *:720-727.* Erratum in: *J Inherit Metab Dis.* ***33****: 93*

6. Ciarmatori S, Kiepe D, Haarmann A, Huegel U, Tönshoff B (2007).
Signaling mechanisms leading to regulation of proliferation and differentiation of the mesenchymal chondrogenic cell line RCJ3.1C5.18 in response to IGF-I.
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7. Kiepe D, Ciarmatori S, Haarmann A, Tönshoff B (2006).
Differential expression of IGF system components in proliferating *vs.* differentiating growth plate chondrocytes: the functional role of IGFBP-5. *Am J Physiol Endocrinol Metab.* ***290****: E363-371*

Reviewer for:

Peritoneal dialysis international
Journal of Inherited Metabolic Disease

Poster presentation:

06/13:	Rare Disease *I*nitiative Zurich (rad*iz*) Summer School: "Expression Studies of Methylmalonyl-CoA Mutase in Mouse and Human Tissue to Investigate the Pathophysiology of Kidney Disease in Methylmalonic Aciduria"
08/13:	9th Symposium Zurich Center for integrative human physiology (ZIHP): "Expression of Methylmalonyl-CoA Mutase: Insights for the Pathophysiology of Kidney Disease in Methylmalonic Aciduria"
09/13:	12th International Congress for Inborn Errors of Metabolism 2013 (ICIEM 2013) "Renal involvement in a patient with cobalamin A type (cblA) methylmalonic aciduria: a 42-year follow-up"
12/14:	Swiss society of nephrology (SSN), Annual meeting, Interlaken: "Pathophysiology of kidney disease in MMA-uria. An up-date".
03/15:	rad*iz* evaluation: "Pathophysiology of kidney disease in MMA-uria."
10/16	Children's Research Center (CRC) retreat: "Insights into the pathophysiology of kidney disease in Methylmalonic aciduria"

Talks:

10/13:	Advanced course on rare metabolic kidney disease, European orphan academy, Rome, 3rd-5th october, "Pathophysiology of kidney disease in Methylmalonic aciduria"
04/15:	Invited talk, Telethon Institute of Genetics and Medicine (TIGEM), Naples, Italy, "Pathophysiology of kidney disease in Methylmalonic aciduria"
13/15:	Several presentations at rad*iz* internal research seminars
09/15:	Talk at the annual symposium of the "Society for the study of inborn errors of metabolism" (SSIEM), Lyon, France
01/16:	Talk at ASMM (Austrian Swiss metabolic meeting) Bern 2016
04/16:	Talk at the 4th Euregio-Meeting, Freiburg, Germany
06/16:	Talk at the 48th European Metabolic Group Meeting (EMG meeting), Freiburg, Germany
03/17	Annual meeting of the German society for inborn errors of metabolism, Fulda, Germany
04/17	"Society for the study of inborn errors of metabolism, academy" (SSIEM academy 17), Lyon

(Financial) support:

09/15:	Attendance of conference in Lyon sponsored by Julius Klaus Stiftung
01/15-06/16	Support from Swiss National Science Foundation (project grant 310030_146490)
01/14-07/17:	Rare disease initiative Zurich (rad*iz*), Clinical Research Priority Program for Rare Diseases, University of Zurich
06/12-12/13:	Zentrum für integrative humane Physiologie
0//12-07/17:	Members of the Clinical Research Priority Program (CRPP) "Rare Diseases Initiative Zurich (radiz)" of the University of Zurich
05/12-05/14:	Funding from Children's University Hospital Heidelberg for collaboration studies